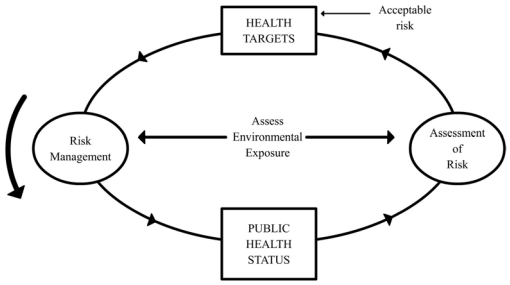

13

Indicators of microbial water quality

Nicholas J. Ashbolt, Willie O.K. Grabow and Mario Snozzi

Current guidelines in the three water-related areas (drinking water, wastewater and recreational water) assess quality, in microbiological terms, by measuring indicator organisms. This chapter looks at the history and examines some of the methods used to assess the microbiological quality of water, highlighting the current limitations and also possible future developments.

13.1 INTRODUCTION

Traditionally, indicator micro-organisms have been used to suggest the presence of pathogens (Berg 1978). Today, however, we understand a myriad of possible reasons for indicator presence and pathogen absence, or vice versa. In short, there is no direct correlation between numbers of any indicator and enteric pathogens (Grabow 1996). To eliminate the ambiguity in the term 'microbial indicator', the following three groups (outlined in Table 13.1) are now recognised:

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- General (process) microbial indicators,
- Faecal indicators (such as *E. coli*)
- Index organisms and model organisms.

Table 13.1. Definitions for indicator and index micro-organisms of public health concern (see Box 13.1 for definitions of microbial groups)

Group	Definition
Process indicator	A group of organisms that demonstrates the efficacy of a process, such as total heterotrophic bacteria or total coliforms for chlorine disinfection.
Faecal indicator	A group of organisms that indicates the presence of faecal contamination, such as the bacterial groups thermotolerant coliforms or *E. coli*. Hence, they only **infer** that pathogens may be present.
Index and model organisms	A group/or species indicative of pathogen presence and behaviour respectively, such as *E. coli* as an index for *Salmonella* and F-RNA coliphages as models of human enteric viruses.

A direct epidemiological approach could be used as an alternative or adjunct to the use of index micro-organisms. Yet epidemiologic methods are generally too insensitive, miss the majority of waterborne disease transmissions (Frost *et al.* 1996) and are clearly not preventative. Nonetheless, the ideal is to validate appropriate index organisms by way of epidemiological studies. A good example is the emerging use of an enterococci guideline for recreational water quality (WHO 1998; Chapter 2 of this volume). Often epidemiologic studies fail to show any relationship to microbial indicators, due to poor design (Fleisher 1990, 1991) and/or due to the widely fluctuating ratio of pathogen(s) to faecal indicators and the varying virulence of the pathogens.

The validity of any indicator system is also affected by the relative rates of removal and destruction of the indicator versus the target hazard. So differences due to environmental resistance or even ability to multiply in the environment all influence their usefulness. Hence, viral, bacterial, parasitic protozoan and helminth pathogens are unlikely to all behave in the same way as a single indicator group, and certainly not in all situations. Furthermore, viruses and other pathogens are not part of the normal faecal microbiota, but are only excreted by infected individuals. Therefore, the higher the number of people contributing to sewage or faecal contamination, the more likely the presence of a range of pathogens. The occurrence of specific pathogens varies further according to their seasonal occurrence (Berg and Metcalf 1978).

In summary, there is no universal indicator, but a number, each with certain characteristics. Therefore, this chapter focuses on elucidating the appropriate

uses for indicator micro-organisms with a view to their role in the management of waterborne microbial risks. To understand the current use of indicators, however, it is necessary to first understand their historical development.

13.2 DEVELOPMENT OF INDICATORS

13.2.1 The coliforms

The use of bacteria as indicators of the sanitary quality of water probably dates back to 1880 when Von Fritsch described *Klebsiella pneumoniae* and *K. rhinoscleromatis* as micro-organisms characteristically found in human faeces (Geldreich 1978). In 1885, Percy and Grace Frankland started the first routine bacteriological examination of water in London, using Robert Koch's solid gelatine media to count bacteria (Hutchinson and Ridgway 1977). Also in 1885, Escherich described *Bacillus coli* (Escherich 1885) (renamed *Escherichia coli* by Castellani and Chalmers (1919)) from the faeces of breast-fed infants.

In 1891, the Franklands came up with the concept that organisms characteristic of sewage must be identified to provide evidence of potentially dangerous pollution (Hutchinson and Ridgway 1977). By 1893, the 'Wurtz method' of enumerating *B. coli* by direct plating of water samples on litmus lactose agar was being used by sanitary bacteriologists, using the concept of acid from lactose as a diagnostic feature. This was followed by gas production, with the introduction of the Durham tube (Durham 1893). The concept of 'coliform' bacteria, those bacteria resembling *B. coli*, was in use in Britain in 1901 (Horrocks 1901). The colony count for bacteria in water, however, was not formally introduced until the first Report 71 (HMSO 1934).

Therefore, the sanitary significance of finding various coliforms along with streptococci and *C. perfringens* (see Box 13.1) was recognised by bacteriologists by the start of the twentieth century (Hutchinson and Ridgway 1977). It was not until 1905, however, that MacConkey (1905) described his now famous MacConkey's broth, which was diagnostic for lactose-fermenting bacteria tolerant of bile salts. Nonetheless, *coli-forms* were still considered to be a heterogeneous group of organisms, many of which were not of faecal origin. The origins of the critical observation that *B. coli* was largely faecal in origin while other coliforms were not, could be claimed by Winslow and Walker (1907).

13.2.1.1 Coliform identification schemes

Various classification schemes for coliforms have emerged. The earliest were those of MacConkey (1909) which recognised 128 different coliform types, while Bergey and Deehan (1908) identified 256. By the early 1920s,

differentiation of coliforms had come to a series of correlations that suggested indole production, gelatin liquefaction, sucrose fermentation and the Voges–Proskauer reaction were among the more important tests for determining faecal contamination (Hendricks 1978). These developments culminated in the IMViC (Indole, Methyl red, Voges–Proskauer and Citrate) tests for the differentiation of so-called faecal coliforms, soil coliforms and intermediates (Parr 1938); these tests are still in use today.

Water sanitary engineers, however, require simple and rapid methods for the detection of faecal indicator bacteria. Hence, the simpler to identify coliform group, despite being less faecal-specific and broader (for which *Escherichia, Klebsiella, Enterobacter* and *Citrobacter* were considered the most common genera) was targeted. One of the first generally accepted methods for coliforms was called the Multiple-Tube Fermentation Test.

Box 13.1. Definitions of key faecal indicator micro-organisms.

Coliforms: Gram-negative, non spore-forming, oxidase-negative, rod-shaped facultative anaerobic bacteria that ferment lactose (with β-galactosidase) to acid and gas within 24–48h at 36±2°C. **Not** specific indicators of faecal pollution.

Thermotolerant coliforms: Coliforms that produce acid and gas from lactose at 44.5± 0.2°C within 24±2h, also known as faecal coliforms due to their role as faecal indicators.

Escherichia coli (E. coli): Thermophilic coliforms that produce indole from tryptophan, but also defined now as coliforms able to produce β-glucuronidase (although taxonomically up to 10% of environmental *E. coli* may not). Most appropriate group of coliforms to indicate faecal pollution from warm-blooded animals.

Faecal streptococci (FS): Gram-positive, catalase-negative cocci from selective media (e.g. azide dextrose broth or m Enterococcus agar) that grow on bile aesculin agar and at 45°C, belonging to the genera *Enterococcus* and *Streptococcus* possessing the Lancefield group D antigen.

Enterococci: All faecal streptococci that grow at pH 9.6, 10° and 45°C and in 6.5% NaCl. Nearly all are members of the genus *Enterococcus*, and also fulfil the following criteria: resistance to 60°C for 30 min and ability to reduce 0.1% methylene blue. The enterococci are a subset of faecal streptococci that grow under the conditions outlined above. Alternatively, enterococci can be directly identified as micro-organisms capable of aerobic growth at 44±0.5°C and of hydrolysing 4-methlumbelliferyl-β-D-glucoside (MUD, detecting β-glucosidase activity by blue florescence at 366nm), in the presence of thallium acetate, nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC, which is reduced to the red formazan) in the specified medium (ISO/FDIS 7899-1 1998).

Sulphite-reducing clostridia (SRC): Gram-positive, spore-forming, non-motile, strictly anaerobic rods that reduce sulphite to H_2S.

Clostridium perfringens: As for SRC, but also ferment lactose, sucrose and inositol with the production of gas, produce a stormy clot fermentation with milk, reduce nitrate, hydrolyse gelatin and produce lecithinase and acid phosphatase. Bonde (1963) suggested that not all SRC in receiving waters are indicators of faecal pollution, hence *C. perfringens* is the appropriate indicator.

Bifidobacteria: Obligately anaerobic, non-acid-fast, non-spore-forming, non-motile, Gram-positive bacilli which are highly pleomorphic and may exhibit branching bulbs (bifids), clubs, coccoid, coryneform, Y and V forms. They are all catalase-negative and ferment lactose (except the three insect species; *B. asteroides, B. indicum* and *B. coryneforme*) and one of the most numerous groups of bacteria in the faeces of warm-blooded animals.
Bacteriophages (phages): These are bacterial viruses and are ubiquitous in the environment. For water quality testing and to model human enteric viruses, most interest in somatic coliphages, male-specific RNA coliphages (F-RNA coliphages) and phages infecting *Bacteroides fragilis*.
Coliphages: Somatic coliphages attack *E. coli* strains via the cell wall and include spherical phages of the family *Microviridae* and various tailed phages in 3 families. The F-RNA coliphages attack *E. coli* strains via the sex pili (F factor) and are single-stranded RNA non-tailed phages in four groups (Table 13.3).
***Bacteroides fragilis* bacteriophages**: These infect one of the most abundant bacteria in the gut, belong to the family *Siphoviridae* with flexible tail (dsDNA, long non-contractile tails, capsids up to 60 nm). Phages to the host strain, *B. fragilis* HSP40 are considered to be human-specific, but phages to *B. fragilis* RYC2056 are more numerous and not human-specific (Puig *et al.* 1999).

13.2.1.2 Most probable number method

In 1914, the first US Public Health Service Drinking Water Standard adopted a bacteriological standard that was applicable to any water supply provided by an interstate common carrier (Wolf 1972). It specified that not more than one out of five 10 ml portions of any sample examined should show the presence of the *B. coli* group by the specified Multiple-Tube Fermentation procedure (now referred to as the Most Probable Number or MPN procedure).

Although this test is simple to perform, it is time-consuming, requiring 48 hours for the presumptive results. There are a number of isolation media each with its bias and the bacteria enriched are not a strict taxonomic group. Hence, the total coliforms can best be described as a range of bacteria in the family *Enterobacteriaceae* varying with the changing composition of the media.

Following presumptive isolation of coliforms, further testing is required for confirmation of the coliform type. During the late 1940s there was a divergence between the UK and US approaches to identifying the thermotolerant or so-called 'faecal' coliforms. In the UK, Mackenzie *et al*. (1948) had shown that atypical fermentors of lactose at 44°C were indole-negative, whereas *E. coli* was indole-positive. Confirmation of *E. coli* with the indole test was undertaken in the UK, but lactose fermentation at 44°C alone was used in the US (Geldreich 1966). Thus over a period of some 50 years, water bacteriologists developed the concept of *B. coli* (later *E. coli*) as the indicator of faecal pollution, but continued to attach significance to the total lactose fermenters,

known variously as 'coli-aerogenes' group, *Escherichia-Aerobacter* group, colon group or generally referred to as the 'total coliforms' group.

The range of non-faecal bacteria represented in the coliform group and the environmental growth of thermophilic (faecal) coliforms *Klebsiella* spp. and *E. coli* (Ashbolt *et al.* 1997; Camper *et al.* 1991) have concerned bacteriologists and sanitary engineers since the 1930s (Committee on Water Supply 1930). At the other extreme, recent outbreaks of cryptosporidiosis in the absence of coliforms (per 100 ml) are well known (Smith and Rose 1998), and many earlier classic failures of coliforms to identify waterborne pathogens have also been reported.

Despite the obvious failings of the total coliform group to indicate health risk from bacterial pathogens, they provide valuable information on process efficiency which is clearly important in relation to health protection.

13.2.1.3 Membrane filtration method

Until the 1950s practical water bacteriology relied almost exclusively, for indicator purposes, on the enumeration of coliforms and *E. coli* based on the production of gas from lactose in liquid media and estimation of most probable numbers using the statistical approach initially suggested by McCrady (1915). In Russia and Germany, however, workers attempted to culture bacteria on membrane filters, and by 1943 Mueller in Germany was using membrane filters in conjunction with Endo-broth for the analysis of potable waters for coliforms (Waite 1985). By the 1950s membrane filtration was a practical alternative to the MPN approach, although the inability to demonstrate gas production with membranes was considered a major drawback (Waite 1985).

The arbitrary definitions adopted for *E. coli* and the related coliforms were all based upon cultural characteristics, including the ability to produce gas from lactose fermentation (HMSO 1969). Hence, the thermotolerant coliforms include strains of the genera *Klebsiella* and *Escherichia* (Dufour 1977), as well as certain *Enterobacter* and *Citrobacter* strains able to grow under the conditions defined for thermotolerant coliforms (Figureras *et al.* 1994; Gleeson and Gray 1996). This phenotypic approach has also resulted in *E. coli* or a related coliform being ignored simply because they failed to ferment lactose, failed to produce gas from lactose or were indole-negative at 44.5°C. The approach had been repeatedly questioned (Waite 1997), and was only resolved in the UK in the 1990s (HMSO 1994).

It has long been recognised that artificial 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 twentieth century (MacConkey 1908), various workers have shown these selective agents inhibit environmentally or oxidatively stressed coliforms.

13.2.1.4 Defined substrate 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*. (1988, 1990, 1991). What has evolved into the Colilert® technique has been shown to correlate very well with the traditional membrane filter and MPN methods when used to test both fresh and marine water (Clark *et al*. 1991; Eckner 1998; Fricker *et al*. 1997; Palmer *et al*. 1993). Furthermore, these enzyme-based methods appear to pick up traditionally non-culturable coliforms (George *et al.* 2000).

These developments have led to further changes in definitions of total coliforms and *E. coli*. In the UK, for example, total coliforms are members of genera or species within the family *Enterobacteriaceae*, capable of growth at 37°C, which possess β-galactosidase (HMSO 1989, 1994). In an international calibration of methods, *E. coli* was enzymatically distinguished by the lack of urease and presence of β-glucuronidase (Gauthier *et al.* 1991). Furthermore, the International Standards Organisation has recently published miniaturised MPN-based methods for coliforms/*E. coli* and enterococci based on the defined substrate approach (ISO/FDIS 1998, 1999).

13.2.2 Faecal streptococci and enterococci

Parallel to the work on coliforms, a group of Gram-positive coccoid bacteria known as faecal streptococci (FS) were being investigated as important pollution indicator bacteria (Houston 1900; Winslow and Hunnewell 1902). Problems in differentiating faecal from non-faecal streptococci, however, initially impeded their use (Kenner 1978). Four key points in favour of the faecal streptococci were:

(1) Relatively high numbers in the excreta of humans and other warm-blooded animals.
(2) Presence in wastewaters and known polluted waters.
(3) Absence from pure waters, virgin soils and environments having no contact with human and animal life.
(4) Persistence without multiplication in the environment.

It was not until 1957, however, with the availability of the selective medium of Slanetz and Bartley (1957) that enumeration of FS became popular. Since then, several media have been proposed for FS and/or enterococci to improve on the specificity.

Taxonomically FS are represented by various *Enterococcus* spp. and *Streptococcus bovis* and *S. equinus* (WHO 1997). Of the faecal streptococci, the preferred indicators of faecal pollution are the enterococci. The predominant intestinal enterococci being *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*. In addition, other *Enterococcus* species and some species of *Streptococcus* (namely *S. bovis* and *S. equinus*) may occasionally be detected. These streptococci however, do not survive for long in water and are probably not enumerated quantitatively. Thus, for water examination purposes enterococci can be regarded as indicators of faecal pollution, although some could occasionally originate from other habitats.

13.2.2.1 Significance of the thermotolerant coliform:faecal streptococci ratio

Geldreich and Kenner (1969) proposed that a faecal coliform:faecal streptococci ratio of four or greater may indicate human pollution, whereas ratios of two or less may indicate animal pollution. There are many factors, however, that can jeopardise the usefulness of this ratio. Foremost are the quicker die-off of coliforms in the environment and different counts from various media used for bacterial isolation (Geldreich 1976). Hence, the use of this ratio is no longer recommended unless very recent faecal pollution is being monitored (Howell *et al*. 1995).

13.2.3 Sulphite-reducing clostridia and other anaerobes

Until bifidobacteria were suggested as faecal indicators (Mossel 1958), *C. perfringens* was the only obligately anaerobic, enteric micro-organism seriously considered as a possible indicator of the sanitary quality of water (Cabelli 1978). Despite the first isolation of bifidobacteria in the late 1800s Tissier 1889) and very high numbers in human faeces (11% of culturable bacteria), their oxygen sensitivity (as with most other strict anaerobes; Loesche 1969) has limited their role as useful faecal indicators in waters (Cabelli 1978; Rhodes and Kator 1999).

The anaerobic sulphite-reducing clostridia (SRC, see Box 13.1) are much less prevalent than bifidobacteria in human faeces, but their spore-forming habit gives them high environmental resistance (Cabelli 1978). *C. perfringens* is the species of clostridia most often associated with the faeces of warm-blooded animals (Rosebury 1962), but is only present in 13–35% of human faeces (Table 13.2).

Although *C. perfringens* has been considered a useful indicator species for over one hundred years (Klein and Houston 1899), its use has been largely limited to Europe, and even then as a secondary indicator mixed in with other SRC (Bonde 1963; HMSO 1969; ISO 1975). The main criticism of the use of *C.*

perfringens as a faecal indicator is its long persistence in the environment, which is considered to be significantly longer than enteric pathogens (Cabelli 1978). Bonde (1963) suggested that all SRC in receiving waters are not indicators of faecal pollution, hence *C. perfringens* is the appropriate indicator.

Table 13.2. Microbial indicators (average numbers per gram wet weight) excreted in the faeces of warm-blooded animals (adapted from Geldreich 1978)

Group	Thermotolerant coliforms	Faecal streptococci	*Clostridium perfringens*	F-RNA Coliphages[b]	Excretion (g/day)
Farm animals					
Chicken	1,300,000	3,400,000	250	1867	182 (71.6)[c]
Cow	230,000	1,300,000	200	84	23,600 (83.3)
Duck	33,000,000	54,000,000	–	13.1	336 (61.0)
Horse	12,600	6,300,000	<1	950	20,000
Pig	3,300,000	84,000,000	3980	4136	2700 (66.7)
Sheep	16,000,000	38,000,000	199,000	1.5	1130 (74.4)
Turkey	290,000	2,800,000	–	–	448 (62.0)
Domestic pets					
Cat	7,900,000	27,000,000	25,100,000		
Dog	23,000,000	980,000,000	251,000,000	2.1	413
Human	13,000,000	3,000,000	1580[a]	<1.0–6.25	150 (77.0)
Ratios in raw sewage	50	5	0.3	1	–

[a] Only 13–35% of humans excrete
[b] F-RNA coliphage data from Calci *et al*. (1998). Note low numbers in human faeces, and only excreted by about 26% of humans, about 60% of domestic animals (including cattle, sheep, horses, pigs, dogs and cats), and 36% of birds (geese and seabirds) (Grabow *et al.* 1995).
[c] Moisture content

13.2.4 Bacteriophages

Viruses which infect bacteria, known as bacteriophages or simply as phages, were first described from the intestinal tract of man in the early 1900s (D'Herelle 1926; Pelczar *et al.* 1988). The use of phages as models for indicating the likely presence of pathogenic enteric bacteria first appeared in the 1930s, and direct correlations between the presence of certain bacteriophages and the intensity of faecal contamination were reported (several references cited by Scarpino 1978).

The evolving role for phages to coliforms, known as coliphages (Box 13.1; Table 13.3) however, has been to model human enteric viruses. The DNA-containing tailed coliphages (T type) and RNA-containing phages that infect via the F-pili (sex factor) (F-RNA coliphages) have been the most used.

Table 13.3. Major groups of indicator coliphages (adapted from Leclerc *et al*. 2000) (ds = double stranded; ss = single stranded)

Gp	Family	Nucleic acid	Tail type	Location of attack	Phage examples	Size (nm)
A	*Myoviridae*	ds DNA	contractile	cell wall	T2, T4, T6 (even numbers)s	95 × 65
B	*Siphoviridae*	ds DNA	Long, non-contractile	cell wall	λ, T5	54
C	*Podoviridae*	ds DNA	Short, non-contractile	cell wall	T7, T3	47
D	*Microviridae*	ss DNA	None, large capsomeres	cell wall	ϕX174, S13	30
E	*Leviviridae*	ss RNA	None, small capsomeres	F+ pili	**Group 1**: MS-2, f2, R-17, JP501 **Group 2**: GA, DS, TH1, BZ13 **Group 3**:Qβ, VK, ST, TW18 **Group 4**: SP, F1, TW19, TW28	20–30
F	*Inoviridae*	ss DNA	No head, flexible filament	F+ pili	SJ2, fd, AF-2,M13	810 × 6

13.2.4.1 Phages in water environments

Studies on the incidence of phages in water environments have been reported from most parts of the world for some decades now. Unfortunately the data are not particularly consistent and comparisons are generally not meaningful. One reason for this is that there are many variables that affect the incidence, survival and behaviour of phages in different water environments, including the densities of both host bacteria and phages, temperature, pH and so on.

Another important reason is the inconsistency in techniques used for the recovery of phages from water environments, and eventual detection and enumeration of the phages. This is not altogether surprising because virology, including phages, is a young and rapidly developing science. Phages can be recovered and detected by many techniques and approaches, and much of this work is still in a research or developmental stage. A major reason for discrepancies in results is the host bacteria used for the detection of various groups of phages. Nonetheless, international collaboration is now leading to meaningful, universally accepted guidelines for the recovery and detection of phages in water environments (such as those produced by the International Organisation for Standardisation).

13.2.5 Faecal sterol biomarkers

The presence of faecal indicator bacteria gives no indication of the source, yet it is widely accepted that human faecal matter is more likely to contain human pathogens than animal faeces. The detection of human enteric viruses is specific, however; the methods are difficult and expensive, and not readily quantifiable. Vivian (1986), in his review of sewage tracers, suggested that using more than one method of determining the degree of sewage pollution would be prudent and advantageous. The use of alternative indicators, in this case faecal sterols as biomarkers, in conjunction with existing microbiological indicators, offers a new way to distinguish sources of faecal contamination and monitor river 'health' (Leeming *et al*. 1998).

Coprostanol has been proposed as a measure of human faecal pollution by a large number of researchers since the late 1960s, however, coprostanol has never really been embraced as a sanitary indicator for sewage pollution because its presence was not considered as indicative of a health risk. However, Leeming *et al.* (1996) showed that herbivores have a different dominant form (24-ethyl coprostanol) and it was later shown that these differences could be exploited to determine the contribution of faecal matter from herbivore and human sources relative to each other (Leeming *et al.* 1998).

13.3 PATHOGEN MODELS AND INDEX MICRO-ORGANISMS

The similar morphology, structure and behaviour of F-RNA coliphages, as well as other phages to that of human enteric viruses, suggests that they should be better models for faecal pollution than the faecal indicator bacteria when human viruses are the likely aetiological agents. The same applies to properties such as removal by water treatment processes and resistance to disinfection processes.

While one would expect a poor correlation of phage numbers to the level of human enteric virus titre (phages are always in sewage, but pathogen numbers vary widely based on human infection), what is important for a model organism is that many phages are as resistant as (human) enteric viruses. Laboratory experiments with individual coliphages confirmed that their survival in natural water environments resembles that of enteric viruses and that some phages are at least as resistant as certain enteric viruses to water environments and to commonly used disinfectant such as chlorine (Grabow 1986; Kott *et al*, 1974; Simkova and Cervenka 1981; Stetler 1984; Yates *et al.* 1985).

The value of phages as models/surrogates for viruses has been applied in the routine monitoring of raw and treated drinking water supplies (Grabow *et al*.

2000), and in the assessment of the efficiency of domestic point-of-use water treatment units (Grabow *et al*. 1999b). While they are useful and meet many of the basic requirements as surrogates for enteric viruses, a number of deficiencies are noted in Box 13.2.

As a result of the deficiencies outlined in Box 13.2 phages cannot be regarded as absolute indicators, models or surrogates for enteric viruses in water environments. This is underlined by the detection of enteric viruses in treated drinking water supplies which yielded negative results in tests for phages, even in presence-absence tests on 500 ml samples of water (Grabow *et al*. 2000). Phages are probably best applied as models/surrogates in laboratory experiments where the survival or behaviour of selected phages and viruses are directly compared under controlled conditions (EPA 1986; Grabow *et al*. 1983, 1999b; Naranjo *et al*. 1997).

In addition to enteric viruses, parasitic protozoa are important disinfection-resistant pathogens. When sewage is the source of these pathogens, the anaerobic spore-forming bacterium *Clostridium perfringens* appears to be a suitable index for enteric viruses and parasitic protozoa (Payment and Franco 1993). Spores of *C. perfringens* are largely of faecal origin (Sorensen *et al*. 1989), and are always present in sewage (about 10^4–10^5 cfu $100ml^{-1}$). Their spores are highly resistant in the environment, and vegetative cells appear not to reproduce in aquatic sediments, which can be a problem with traditional indicator bacteria (Davies *et al*. 1995).

Like spores to *C. perfringens*, *Bacillus* spp. spores can also be used as models for parasitic protozoan cysts or oocysts removal by water treatment (Rice *et al*. 1996). Furthermore, since vegetative bacterial cells are inadequate models for disinfection, phages or clostridial spores may provide useful models (Tyrrell *et al*. 1995; Venczel *et al*. 1997).

Box 13.2. Limitations of phages.

Phages are excreted by a certain percentage of humans and animals all the time while viruses are excreted only by infected individuals for a short period of time. The excretion of viruses heavily depends on variables such as the epidemiology of various viruses, outbreaks of viral infections and vaccination against viral infections (Grabow *et al*. 1999a). Consequently there is no direct correlation between numbers of phages and viruses excreted by humans (Borrego *et al*. 1990; Grabow *et al*. 1993; Vaughn and Metcalf 1975).

Methods for somatic coliphages detect a wide range of phages with different properties (Gerba 1987; Yates *et al*. 1985).

At least some somatic coliphages may replicate in water environments (Borrego *et al*. 1990; Grabow *et al.* 1984; Seeley and Primrose 1982).

Enteric viruses have been detected in water environments in the absence of coliphages (Deetz *et al*. 1984; Montgomery 1982; Morinigo *et al*. 1992).

Human enteric viruses associated with waterborne diseases are excreted almost exclusively by humans (Grabow 1996). Phages used as models/surrogates in water quality assessment are excreted by humans and animals. In fact, the faeces of animals such as cows and pigs generally contains higher densities of coliphages than that of humans (Furuse *et al*. 1983; Osawa *et al*. 1981), and the percentage of many animals that excrete phages tends to be higher than for humans (Grabow *et al*. 1993, 1995).

The microbiota of the gut, diet, and physiological state of animals seems to affect the numbers of coliphages in faeces. Osawa *et al.* (1981) reported that stools from zoo animals contained a higher percentage of positive tests for phages than those from domestic farm animals.

The composition and numbers of phages excreted by humans is variable. Patients under antibiotic treatment were found to excrete lower numbers of phages than comparable patients and healthy individuals not exposed to antibiotics (Furuse *et al*. 1983).

Differences between phages and enteric viruses are also reflected by differences in the efficiency of adsorption-elution techniques for their recovery. These differences are due to differences in adsorption properties, which have major implications for behaviour in water environments and at least some treatment processes.

13.4 EMERGING MICROBIOLOGICAL METHODS

13.4.1 Fast detections using chromogenic substances

The time required to perform tests for indicator organisms has stimulated research into more reliable and faster methods. One result is the use of chromogenic compounds, which may be added to the conventional or newly devised media used for the isolation of the indicator bacteria. These chromogenic substances are modified either by enzymes (which are typical for the respective bacteria) or by specific bacterial metabolites. After modification the chromogenic substance changes its colour or its fluorescence, thus enabling easy detection of those colonies displaying the metabolic capacity. In this way these substances can be used to avoid the need for isolation of pure cultures and confirmatory tests. The time required for the determination of different indicator bacteria can be cut down to between 14 to 18 hours.

13.4.2 Application of monoclonal and polyclonal antibodies

Antibodies (glycoproteins produced by mammals as part of their defence system against foreign matter) possess highly specific binding and recognition domains that can be targeted to specific surface structures of a pathogen (antigen).

Immunological methods using antibodies are widely used to detect pathogens in clinical, agricultural and environmental samples. Antisera or polyclonal antibodies, the original source of immune reagents, are obtained from the serum of immunised animals (typically rabbits or sheep). Monoclonal antibodies which are produced *in vitro* by fusing plasma cells of an immunised animal (usually a mouse or rat) with a cell line that grows continuously in culture (so that the fused cells will grow continuously and secrete only one kind of antibody molecule (Goding 1986)), can be much better standardised (Torrance 1999).

Such monoclonal antibodies have been successfully used for the detection of indicator bacteria in water samples (Hübner *et al.* 1992; Obst *et al.* 1994). In these studies the water sample was subjected to a precultivation in a selective medium. In this way the complication of detecting dead cells was avoided. Another option for the detection of 'viable' indicators is the combination of immunofluorescence with a respiratory activity compound. This approach has been described for the detection of *E. coli* O157:H7, *S. typhimurium* and *K. pneumoniae* in water (Pyle *et al*. 1995). Detection of *Legionella* from water samples has also been achieved with antibodies (Obst *et al.* 1994; Steinmetz *et al.* 1992). In general, immunological methods can easily be automated in order to handle high sample numbers.

Antibody technology is often used in medicine with enzyme amplification (ELISA – enzyme linked immunosorbent assay), to allow the development of an antigen signal readable by the naked eye. Such an approach is under development for the rapid identification of coliform microcolonies (Sartory and Watkins 1999). As always with immunological techniques, the specificity of the reagents and optimisation of their use is paramount. Although total coliforms are a broad group and likely to be unsuitable immunological targets in environmental waters, *E. coli* could be identified from other coliforms.

Until reliable index organisms are identified for the parasitic protozoa C*ryptosporidium* and *Giardia*, their detection is also relevant when describing methods for important faecal organisms. Current methods for their detection rely on antibodies to assist in the microscopic identification amongst other environmental particles (Graczyk *et al.* 1996). In addition, magnetic beads coated with antibodies are used for concentration and separation of the oocysts and cysts (Rochelle *et al*. 1999) as described below for immunomagnetic separation (IMS) methods.

13.4.3 IMS/culture and other rapid culture-based methods

Immunomagnetic separation offers an alternative approach to rapid identification of culturable and non-culturable micro-organisms (Safarik *et al*.

1995). The principles and application of the method are simple, but rely on suitable antibody specificity under the conditions of use. Purified antigens are typically biotinylated and bound to streptoavidin-coated paramagnetic particles (e.g. Dynal™ beads). The raw sample is gently mixed with the immunomagnetic beads, then a specific magnet is used to hold the target organisms against the wall of the recovery vial, and non-bound material is poured off. If required, the process can be repeated, and the beads may be removed by simple vortexing. Target organisms can then be cultured or identified by direct means.

The IMS approach may be applied to recovery of indicator bacteria from water, but is possibly more suited to replace labour-intensive methods for specific pathogens. An example is the recovery of *E. coli* O157 from water (Anon 1996a). Furthermore, *E. coli* O157 detection following IMS can be improved by electrochemiluminescence detection (Yu and Bruno 1996).

13.4.4 Gene sequence-based methods

Advances in molecular biology in the past 20 years have resulted in a number of new detection methods, which depend on the recognition of specific gene sequences. Such methods are usually rapid and can be tailored to detect specific strains of organisms on the one hand or groups of organisms on the other. The methods have a substantial potential for future application in the field of drinking water hygiene (Havelaar 1993). An international expert meeting in Interlaken concluded (OECD 1999) that the application of molecular methods has to be considered in a framework of a quality management for drinking water. The new methods will influence epidemiology and outbreak investigations more than the routine testing of finished drinking water.

13.4.4.1 PCR (polymerase chain reaction)

With the polymerase chain reaction and two suitable primer sequences (fragments of nucleic acid that specifically bind to the target organism) trace amounts of DNA can be selectively multiplied. In principle, a single copy of the respective sequence in the assay can produce over a million-fold identical copies, which then can be detected and further analysed by different methods.

One problem with PCR is that the assay volume is in the order of some micro-litres, whereas the water sample volume is in the range of 100–1000 ml. Bej *et al*. (1991) have published a filtration method to concentrate the sample, but another problem is that natural water samples often contain inhibitory substances (such as humic acids and iron) that concentrate with the nucleic

acids. Hence, it is critical to have positive (and negative) controls with each environmental sample PCR to check for inhibition and specificity.

It may also be critical to find out whether the signal obtained from the PCR is due to naked nucleic acids or living or dead micro-organisms (Toze 1999). One solution has been established by using a three-hour pre-incubation period in a selective medium so that only growing organisms are detected (Frahm *et al.* 1998). Other options under development include targeting short-lived nucleic acids such as messenger or ribosomal RNA (Sheridan *et al*. 1998).

A most important advantage of PCR is that the target organism(s) do not need to be culturable. A good example is the specific detection of human *Bacteroides* spp. to differentiate human faecal pollution from that of other animals (Kreader 1995).

13.4.4.2 FISH (fluorescence in situ hybridisation)

This detection method uses gene probes with a fluorescent marker, typically targeting the 16S ribosomal RNA (16S rRNA) (Amann *et al*. 1995). Concentrated and fixed cells are permeabilised and mixed with the probe. Incubation temperature and addition of chemicals can influence the stringency of the match between the gene probe and the target sequence. Since the signal of a single fluorescent molecule within a cell does not allow detection, target sequences with multiple copies in a cell have to be selected (e.g. there are 10^2–10^4 copies of 16S rRNA in active cells). A number of FISH methods for the detection of coliforms and enterococci have been developed (Fuchs *et al*. 1998; Meier *et al*. 1997; Patel *et al*. 1998).

Although controversial for many pathogens, low-nutrient environments may result in cells entering a non-replicative viable but non-culturable (VBNC) state (Bogosian *et al*. 1998). Such a state may not only give a false sense of security when reliant on culture-based methods, but may also give the organisms additional protection (Caro *et al*. 1999; Lisle *et al*. 1998). An indication of VBNC *Legionella pneumophila* cell formation was given by following decreasing numbers of bacteria monitored by colony-forming units, acridine orange direct count, and hybridisation with 16S rRNA-targeted oligonucleotide probes (Steinert *et al*. 1997). It was concluded that FISH detection-based methods may better report the presence of infective pathogens and viable indicator bacteria.

13.4.5 Future developments

The future holds numerous possibilities for the detection of indicator and pathogen index organisms. On the horizon are methods based on microarrays and biosensors. Biosensors in the medical area have largely been based on

antibody technology, with the antigen triggering a transducer or linking to an enzyme amplification system. Biosensors based on gene recognition, however, look very promising in the microarray format for detecting micro-organisms.

Microarrays using DNA/RNA probe-based rRNA targets may be coupled to adjacent 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 ($1cm^2$) of a conventional microtiter plate (96 well). The complete assay with quantification took less than a minute.

DNA sensing protocols, based on different modes of nucleic acid interaction, possess an enormous potential for environmental monitoring. Carbon strip or paste electrode transducers, supporting the DNA recognition layer, are used with a highly sensitive chronopotentiometric transduction of the DNA analyte recognition event. Pathogens targeted to date include *Mycobacterium tuberculosis, Cryptosporidium parvum* and HIV-1 (Vahey *et al*. 1999; Wang *et al*. 1997a,b).

13.5 THE CURRENT APPLICABILITY OF FAECAL INDICATORS

Many members of the total coliform group and some so-called faecal coliforms (e.g. species of *Klebsiella* and *Enterobacter*) are not specific to faeces, and even *E. coli* has been shown to grow in some natural aquatic environments (Ashbolt *et al*. 1997; Bermudez and Hazen 1988; Hardina and Fujioka 1991; Niemi *et al*. 1997; Solo-Gabriele *et al*. 2000; Zhao *et al*. 1997). Hence, the primary targets representing faecal contamination in temperate waters are now considered to be *E. coli* and enterococci. For tropical waters/soils, where *E. coli* and enterococci may grow, alternative indicators such as *Clostridium perfringens* may be preferable.

Numerous epidemiological studies of waterborne illness in developed countries indicate that the common aetiological agents are more likely to be viruses and parasitic protozoa than bacteria (Levy *et al*. 1998). Given the often lower persistence of vegetative cells of the faecal bacteria compared to the former agents, it is not surprising that poor correlations have been reported between waterborne human viruses or protozoa and thermotolerant coliforms (Kramer *et al*. 1996). Such a situation is critical to understand, as evident from recent drinking water outbreaks where coliform standards were met (Craun *et al*. 1997; Marshall *et al*. 1997). Nonetheless, water regulatory agencies have yet to come to terms with the inherent problems resulting from reliance on faecal indicator bacteria as currently determined.

Fortunately, new index organisms for some pathogens look promising as performance organisms in the HACCP-type management approaches (see Chapter 12). Examples of such index organisms are *C. perfringens* and the phages. *C. perfringens* for parasitic protozoa, but only if derived from human faecal contamination (Ferguson *et al.* 1996; Payment and Franco 1993). Their resistance to disinfectants may also be an advantage for indexing disinfectant-resistant pathogens. In Europe, the European Union (EU) recommends the absence of *C. perfringens* in 100ml as a secondary attribute to drinking waters (EU 1998), while in Hawaii, levels are laid down for marine and fresh waters (Anon 1996b). Also F-RNA coliphages or *Bacteroides fragilis* bacteriophages are preferred to assess the removal or persistence of enteric viruses (Calci *et al*. 1998; Puig *et al.* 1999; Shin and Sobsey 1998; Sinton *et al*. 1999). As these index organisms are relatively untested worldwide, extensive trials are necessary before their general acceptance in microbial risk assessment. It should be noted that useful index organisms in one system are not necessarily of value in a different environment.

A further confusion over the use of indicator organisms arises from the fact that some indicator strains are also pathogens. This is perhaps best illustrated by the toxigenic *E. coli* strains (Ohno *et al.* 1997). *E. coli* O157:H7 has been responsible for illness to recreational swimmers (Ackman *et al.* 1997; Keene *et al.* 1994; Voelker 1996) and several deaths have been documented through food- and waterborne outbreaks (HMSO 1996; Jones and Roworth 1996). Such toxigenic *E. coli* are also problematic to detect, as they may form viable but non-culturable cells in water (Kogure and Ikemoto 1997; Pommepuy *et al.* 1996).

13.6 IMPLICATIONS FOR INTERNATIONAL GUIDELINES AND NATIONAL REGULATIONS

Indicators have traditionally played a very important role in guidelines and national standards. Increasingly, however, they are being seen as an adjunct to management controls, such as sanitary surveys, and there is a move away from a specified indicator level end product. In other words, indicators are being replaced by on-line analyses (say for chlorine residual or particle sizes) at critical control points (Chapter 12).

A single indicator or even a range of indicators is unlikely to be appropriate for every occasion and therefore it is useful to tailor indicator choice to local circumstances when translating international guidelines into national standards. Additionally, with the change in management paradigm, more indicators of

process efficiency are required rather than reliance on the 'old-style' faecal indicators.

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