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Detection and enumeration of coliforms in drinking water: current methods and emerging approaches

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Abstract

The coliform group has been used extensively as an indicator of water quality and has historically led to the public health protection concept. The aim of this review is to examine methods currently in use or which can be proposed for the monitoring of coliforms in drinking water. Actually, the need for more rapid, sensitive and specific tests is essential in the water industry. Routine and widely accepted techniques are discussed, as are methods which have emerged from recent research developments. Approved traditional methods for coliform detection include the multiple-tube fermentation (MTF) technique and the membrane filter (MF) technique using different specific media and incubation conditions. These methods have limitations, however, such as duration of incubation, antagonistic organism interference, lack of specificity and poor detection of slowgrowing or viable but non-culturable (VBNC) microorganisms. Nowadays, the simple and inexpensive membrane filter technique is the most widely used method for routine enumeration of coliforms in drinking water. The detection of coliforms based on specific enzymatic activity has improved the sensitivity of these methods. The enzymes β -D galactosidase and β -D glucuronidase are widely used for the detection and enumeration of total coliforms and Escherichia coli, respectively. Many chromogenic and fluorogenic substrates exist for the specific detection of these enzymatic activities, and various commercial tests based on these substrates are available. Numerous comparisons have shown these tests may be a suitable alternative to the classical techniques. They are, however, more expensive, and the incubation time, even though reduced, remains too long for same-day results. More sophisticated analytical tools such as solid phase cytometry can be employed to decrease the time needed for the detection of bacterial enzymatic activities, with a low detection threshold. Detection of coliforms by molecular methods is also proposed, as these methods allow for very specific and rapid detection without the need for a cultivation step. Three molecular-based methods are evaluated here: the immunological, polymerase chain reaction (PCR) and in-situ hybridization (ISH) techniques. In the immunological approach, various antibodies against coliform bacteria have been produced, but the application of this technique often showed low antibody specificity. PCR can be used to detect coliform bacteria by means of signal amplification: DNA sequence coding for the *lacZ* gene (β -galactosidase gene) and the *uidA* gene (β -D glucuronidase gene) has been used to detect total coliforms and E. coli, respectively. However, quantification with PCR is still lacking in precision and necessitates extensive laboratory work. The FISH technique involves the use of oligonucleotide probes to detect complementary sequences inside specific cells. Oligonucleotide probes designed specifically for regions of the 16S RNA molecules of Enterobacteriaceae can be used for microbiological quality control of drinking water samples. FISH should

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be an interesting viable alternative to the conventional culture methods for the detection of coliforms in drinking water, as it provides quantitative data in a fairly short period of time (6 to 8 h), but still requires research effort. This review shows that even though many innovative bacterial detection methods have been developed, few have the potential for becoming a standardized method for the detection of coliforms in drinking water samples. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Public and environmental health protection requires safe drinking water, which means that it must be free of pathogenic bacteria. Among the pathogens disseminated in water sources, enteric pathogens are the ones most frequently encountered. As a consequence, sources of fecal pollution in waters devoted to human activity must be strictly controlled. Entero-pathogens, such as *Escherischia coli* O157:H7, are generally present at very low concentrations in environmental waters within a diversified microflora. Complex methods are required to detect them, and these are extremely time-consuming.

Most coliforms are present in large numbers among the intestinal flora of humans and other warm-blooded animals, and are thus found in fecal wastes. As a consequence, coliforms, detected in higher concentrations than pathogenic bacteria, are used as an index of the potential presence of entero-pathogens in water environments. The use of the coliform group, and more specifically E. coli, as an indicator of microbiological water quality dates from their first isolation from feces at the end of the 19th century. Coliforms are also routinely found in diversified natural environments, as some of them are of telluric origin, but drinking water is not a natural environment for them. Their presence in drinking water must at least be considered as a possible threat or indicative of microbiological water quality deterioration. Positive total coliform samples in a treated water which is usually coliform-free may indicate treatment ineffectiveness, loss of disinfectant, breakthrough (McFeters et al., 1986), intrusion of contaminated water into the potable water supply (Geldreich et al., 1992; Clark et al., 1996) or regrowth problems (LeChevallier, 1990) in the distribution system, and, as a consequence, should not be tolerated.

The use of the coliform group as an indicator of the possible presence of enteric pathogens in aquatic sys-

tems has been a subject of debate for many years. Many authors have reported waterborne disease outbreaks in water meeting the coliform regulations (Payment et al., 1991; Moore et al., 1994; MacKenzie et al., 1994; Gofti et al., 1999). However, the purpose of this review is not to discuss the indicator concept, but rather to identify methods currently in use or which can be proposed for the monitoring of coliforms in drinking water. The need for more rapid and sensitive tests is constant in the water industry, with the ultimate goal being the continuous on-line monitoring of water leaving treatment plants.

1.1. What are coliforms?

The coliform group includes a broad diversity in terms of genus and species, whether or not they belong to the Enterobacteriaceae family. Most definitions of coliforms are essentially based on common biochemical characteristics. In *Standard Methods for the Examination of Water and Wastewater* (Part 9221 and 9222; APHA et al., 1998), coliform group members are described as:

- 1. all aerobic and facultative anaerobic, Gramnegative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35 °C (multiple-tube fermentation technique; Section 3.1) or
- 2. all aerobic and many facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that develop a red colony with a metallic sheen within 24 h at 35 °C on an Endo-type medium containing lactose (membrane filter technique; Section 3.2).

The definition of members of the coliform group has recently been extended to include other characteristics, such as β -D-galactosidase-positive reactions (Part 9223; APHA et al., 1998) (enzyme substrate test, Section 4.2). The search for β -galactosidasepositive and β -galactoside-permease-positive organisms also permits a confirmation step for lactose fermentation, when the multiple-tube fermentation technique is used. The cytochrome-oxidase test is also used as a confirmation test to eliminate some bacteria of the *Aeromonas* or *Pseudomonas* genera that would ferment lactose.

The definition of coliform bacteria differs slightly depending on the country or on the organization in charge of the microbiological monitoring regulations. In Canada, the definition is the same as in the US, and differs in some European countries. For example, the French Standardization Association (NFT90-413 and NFT90-414; AFNOR, 1990), which can be considered as a representative model for European legislation, defines total coliforms (TC) as:

• rod-shaped, non-spore-forming, Gram-negative, oxidase-negative, aerobic or facultative anaerobic bacteria that are able to grow in the presence of bile salts or other replacement surface active agents having an analogous growth inhibitory effect and that ferment lactose with gas and acid (or aldehyde) production within 48 h at 37 ± 1 °C.

AFNOR (1990) goes further by defining other coliform groups, including the thermotolerant coliforms (also called fecal coliforms, FC) and, more specifically, *E. coli*:

• thermotolerant coliforms have the same fermentation properties as total coliforms (TC) but at a temperature of 44 ± 0.5 °C.

• *E. coli* is a thermotolerant coliform which, among other things, produces indole from tryptophane at a temperature of 44 ± 0.5 °C, gives a positive methyl red test result, is unable to produce acetyl-methyl carbinol and does not use citrate as its sole carbon source.

The use of the coliform group as an indicator of fecal contamination is subject to strict governmental regulations (Table 1). *E. coli* is the most common coliform among the intestinal flora of warm-blooded animals and its presence might be principally associated with fecal contamination. No *E. coli* are therefore allowed in drinking water.

The US Environmental Protection Agency (EPA) has approved several methods for coliform detection: the multiple-tube fermentation technique, the membrane filter technique and the presence/absence test (including the ONPG-MUG test). AFNOR (1990) has approved the multiple-tube fermentation technique and the membrane filter technique.

These methods have limitations, such as duration of incubation, antagonistic organisms interference, lack of specificity to the coliform group and a weak level of detection of slow-growing or stressed coliforms. Indeed, depending on the environmental system, only a small portion (0.1-15%) of the total bacterial population can be enumerated by cultivation-based methods (Amann et al., 1990). The proportion of non-culturable bacteria may be affected by unfavorable conditions for bacterial growth during culturing or by their entry into viable or active but non-culturable states (VBNC or ABNC) (Roszak and

Table 1

Some existing bacterial contamination regulations and guidelines for drinking water

	Total coliform	E. coli	Monitoring requirements		
			Population	Samples/months	
United States ^a	0/100 ml (95%) a consecutive sample from the same site must be coliform-free	0/100 ml (100%)		$\approx 1/1000$ inhabitants	
Canada ^b	0/100 ml (90%) none should contain more than 10 CFU/100 ml	0/100 ml (100%)	< 5000 5000-9000	4 samples/month 1/1000 inhabitants	
World Health Organization ^c	same site must be coliform-free 0/100 ml (95%)	0/100 ml (100%)	× 9000	90+ (1/10,000 innabitants)	

^a US Environmental Protection Agency, 1990.

^b Ministère de la santé, 1996.

^c World Health Organisation, 1994.

Colwell, 1987; Joux and Lebaron, 2000; Colwell and Grimes, 2000).

2. Objectives

Since drinking waters constitute oligotrophic systems, the lack of sensitivity of cultivation methods in the detection of stressed and starved bacterial cells can generate serious limitations due to contamination-level underestimation. There exist other methods which may be used for coliform detection, and these are in various states of development and application. This review describes the principles and the usual protocols of the classical methods, as well as some innovative methods and emerging approaches. The applicability of the various methods to the detection of coliforms in an oligotrophic environment like drinking water is also evaluated based on their advantages and disadvantages. Criteria such as detection limit and sensitivity of the method, time required to obtain a result and laboratory outlays (including skill, labor and cost) are also discussed.

3. Classical methods

3.1. Multiple-tube fermentation technique

The technique of enumerating coliforms by means of multiple-tube fermentation (MTF) has been used for over 80 years as a water quality monitoring method. The method consists of inoculating a series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 h of incubation at 35 °C constitutes a positive presumptive reaction. Both lactose and lauryl tryptose broths can be used as presumptive media, but Seidler et al. (1981) and Evans et al. (1981) have obtained interference, with high numbers of non-coliform bacteria, using lactose broth. All tubes with a positive presumptive reaction are subsequently subjected to a confirmation test. The formation of gas in a brilliant green lactose bile broth fermentation tube at any time within 48 h at 35 °C constitutes a positive confirmation test. The fecal coliform test (using an EC medium) can be applied to determine TC that are FC (APHA et al., 1998): the

production of gas after 24 h of incubation at 44.5 °C in an EC broth medium is considered as a positive result.

The results of the MTF technique are expressed in terms of the most probable number (MPN) of microorganisms present. This number is a statistical estimate of the mean number of coliforms in the sample. As a consequence, this technique offers a semi-quantitative enumeration of coliforms. Nevertheless, the precision of the estimation is low and depends on the number of tubes used for the analysis: for example, if only 1 ml is examined in a sample containing 1 coliform/ml, about 37% of 1-ml tubes may be expected to yield negative results because of the random distribution of the bacteria in the sample. But, if five tubes, each with 1 ml sample, are used, a negative result may be expected less than 1% of the time (APHA et al., 1998).

Many factors may significantly affect coliform bacteria detection by MTF, especially during the presumptive phase. Interference by high numbers of noncoliform bacteria (Seidler et al., 1981; Evans et al., 1981; Means and Olson, 1981), as well as the inhibitory nature of the media (McFeters et al., 1982), have been identified as factors contributing to underestimates of coliform abundance. The MTF technique lacks precision in qualitative and quantitative terms. The time required to obtain results is higher than with the membrane filter technique that has replaced the MTF technique in many instances for the systematic examination of drinking water. However, this technique remains useful, especially when the conditions do not allow the use of the membrane filter technique, such as turbid or colored waters.

MTF is easy to implement and can be performed by a technician with basic microbiological training, but the method can become very tedious and laborintensive since many dilutions have to be processed for each water sample. However, it is also relatively inexpensive, as it requires unsophisticated laboratory equipment. Nevertheless, this method is extremely time-consuming, requiring 48 h for presumptive results, and necessitates a subculture stage for confirmation which could take up to a further 48 h.

3.2. Membrane filter technique

The membrane filter (MF) technique is fully accepted and approved as a procedure for monitoring

drinking water microbial quality in many countries. This method consists of filtering a water sample on a sterile filter with a 0.45-µm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter.

Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples (Grabow and du Preez, 1979; Rice et al., 1987). Among these, the most widely used for drinking water analysis are the m-Endo-type media in North America (APHA et al., 1998) and the Tergitol-TTC medium in Europe (AFNOR, 1990). Coliform bacteria form red colonies with a metallic sheen on an Endo-type medium containing lactose (incubation 24 h at 35 °C for TC) or yellow-orange colonies on Tergitol-TTC media (incubation 24 and 48 h at 37 and 44 °C for TC and FC, respectively). Other media, such as MacConkey agar and the Teepol medium, have been used in South Africa and Britain. However, comparisons among the media have shown that m-Endo agar yielded higher counts than MacConkey or Teepol agar (Grabow and du Preez, 1979). To enumerate FC, the APHA et al. (1998) proposes that filters be incubated on an enriched lactose medium (m-FC) at a temperature of 44.5 °C for 24 h. Because of the elevated incubation temperature and the addition of rosolic acid salt reagent, few nonfecal coliform colonies develop on the m-FC medium (APHA et al., 1998).

Enumeration of TC by membrane filtration is not totally specific. When MF is associated with m-Endo media containing lactose, atypical colonies which are dark red, mucoid or nucleated and without a metallic sheen may occasionally appear. Atypical blue, pink, white or colorless colonies lacking sheen are not considered as TC by this technique (APHA et al., 1998). Furthermore, typical colonies with a sheen may be produced occasionally by non-coliform bacteria and, conversely, atypical colonies (dark red or nucleated colonies without sheen) may sporadically be coliforms. Coliform verification is therefore recommended for both types of colonies (APHA et al., 1998).

With the acceptance of MF as a technique of choice for drinking water monitoring (APHA et al., 1998; AFNOR, 1990), questions regarding interference with coliform detection and the accuracy of the enumeration have arisen. The presence of high num-

bers of background heterotrophic bacteria was shown to decrease coliform recovery by MF (Clark, 1980; Burlingame et al., 1984). Excessive crowding of colonies on m-Endo media has been associated with a reduction in coliform colonies producing the metallic sheen (Hsu and Williams, 1982; Burlingame et al., 1984).

The predominant concern about MF is its inability to recover stressed or injured coliforms. A number of chemical and physical factors involved in drinking water treatment, including disinfection, can cause sublethal injury to coliform bacteria, resulting in a damaged cell unable to form a colony on a selective medium. Exposure of bacteria to products like chlorine may result in injury and increased sensitivity to bile salts or to the replacement surface-active agents (sodium desoxycholate or Tergitol 7) contained in some selective media. Some improvements in the method have increased detection of injured coliform bacteria, including the development of m-T7 medium formulated specifically for the recovery of stressed coliforms in drinking water (LeChevallier et al., 1983). Evaluation on routine drinking (LeChevallier et al., 1983; McFeters et al., 1986) and surface (McFeters et al., 1986; Freier and Hartman, 1987) water samples showed higher coliform recovery on the m-T7 medium as compared with that on the m-Endo medium. However, m-T7 may not be as efficient when stressing agents other than chlorine are involved. Rice et al. (1987) achieved no significant difference in coliform recovery on m-T7 compared with m-Endo LES from monochloraminated samples, and Adams et al. (1989) found that the m-T7 medium performed no better than the m-Endo medium in enumerating E. coli and C. freundii cells exposed to ozone.

Other authors have suggested that chlorination affects various functions of coliform bacteria activity, such as catalase enzymatic activity (Calabrese and Bissonnette, 1990; Sartory, 1995). Metabolically active bacteria produce hydrogen peroxide (H_2O_2), which is usually rapidly degraded by the catalase. Injured coliforms with reduced catalase activity accumulate toxic hydrogen peroxide, to which they are extremely sensitive. Calabrese and Bissonnette (1990) reported an increase in coliform recovery on m-Endo and m-T7 media, as well as an increase in *E. coli* recovery on an m-FC medium when these media were supplemented with catalase, sodium pyruvate or both. Sartory (1995)

The high number of modified media in use is a reflection of the fact that no universal medium currently exists which allows optimal enumeration of various coliform species originating from different environments and present in a wide variety of physiological states. A significant advantage of the MF technique over the MTF method is that with MF, the examination of larger volumes of water is feasible, which leads to greater sensitivity and reliability. MF also offers a quantitative enumeration comparatively to the semiquantitative information given by the MTF method. MF is a useful technique for the majority of water quality laboratories as it is a relatively simple method to use. Many samples can be processed in a day with limited laboratory equipment by a technician with basic microbiological training. Nevertheless, since this method is not sufficiently specific, a confirmation stage is needed, which could take a further 24 h after the first incubation period on selective media. This is why improvements to MF methods based on the enzymatic properties of coliforms have been proposed (see Section 4.3).

4. Enzymatic methods

4.1. General principles

The biochemical tests used for bacterial identification and enumeration in classical culture methods are generally based on metabolic reactions. For this reason, they are not fully specific, and many additional tests are sometimes required to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted. Moreover, reactions are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigation for many years now.

 β -D-glucuronidase is an enzyme which catalyzes the hydrolysis of β -D-glucopyranosiduronic derivatives into their corresponding aglycons and D-glucuronic acid. Although this bacterial enzyme was discovered first in E. coli, its relative specificity for identifying this microorganism was not apparent until Kilian and Bulow (1976) surveyed the Enterobacteriaceae and reported that glucuronidase activity was mostly limited to E. coli. The prevalence of this enzyme and its utility in the detection of E. coli in water were later reviewed by Hartman (1989). β-Dglucuronidase-positive reactions were observed in 94-96% of the E. coli isolates tested (Kilian and Bulow, 1976; Feng and Hartman, 1982; Edberg and Kontnick, 1986; Kaspar et al., 1987), while Chang et al. (1989) found a higher proportion of B-D-glucuronidase-negative E. coli (a median of 15% from E. coli isolated from human fecal samples). In contrast, β -Dglucuronidase activity is less common in other Enterobacteriaceae genus, such as Shigella (44 to 58%), Salmonella (20 to 29%) and Yersinia strains and in Flavobacteria (Kilian and Bulow, 1976; Massanti et al., 1981; Feng and Hartman, 1982; Frampton and Restaino, 1993). β -D-galactosidase, catalyzes the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group within the Enterobacteriaceae family. The use of the B-D-glucuronidase and B-D-galactosidase activities for the detection and enumeration of E. coli and TC, respectively, are reviewed here.

Chromogenic and fluorogenic substrates produce color and fluorescence, respectively upon cleavage by a specific enzyme. These substrates have been used to detect the presence or the activity of specific enzymes in aquatic systems (Chröst, 1991). Several authors have reviewed fluorogenic and chromogenic substrates used for bacterial diagnostics (Bascomb, 1987; Manafi et al., 1991). They noted that the use of these substrates has led to improved accuracy and faster detection. Methods for detection or enumeration may be performed in a single medium, thus bypassing the need for a time-consuming isolation procedure prior to identification. To detect the presence of β -D-glucuronidase in *E. coli*, the following chromogenic substrates were used: indoxyl-\beta-D-glucuronide (IBDG) (Brenner et al., 1993), the phenolphthalein-mono-\beta-D-glucuronide complex (Bütle and Reuter, 1989) and 5-bromo-4-chloro-3-indolyl-B-Dglucuronide (X-Glu) (Watkins et al., 1988). Most frequently, the fluorogenic substrate 4-methylumbelliferyl-\beta-D-glucuronide (MUGlu) was used (Dahlen and Linde, 1973; Feng and Hartman, 1982). Chromogenic

substrates such as *o*-nitrophenyl- β -D-galactopyranoside (ONPG), *p*-nitrophenyl- β -D-galactopyranoside (PNPG), 6-bromo-2-naphtyl- β -D-galactopyranoside (Bürger, 1967) and 5-bromo-4-chloro-3-indolyl- β -Dgalactopyranoside (X-Gal) (Ley et al., 1993) were used to detect the presence of β -D-galactosidase produced by coliforms, as well as the fluorogenic substrate 4-methylumbelliferyl- β -D-galactopyranoside (MUGal).

4.2. Presence/absence techniques and enumeration by multi-tube techniques using enzymatic methods

The incorporation of MUGlu into lauryl tryptose broth used as the medium for the multi-tube fermentation (MTF) technique was first proposed for rapid detection and immediate confirmation of *E. coli* in food and water samples (Feng and Hartman, 1982). The presence of methylumbelliferone due to the hydrolysis of MUGlu (positive samples) was detected by exposure to long-wave UV light and visualization of blue-white fluorescence. Some authors have suggested using a spectrofluorometer (Park et al., 1995) or a spectrophotometer (Standridge et al., 1992; Apte et al., 1995) to decrease the threshold of fluorescence detection and thus reduce the incubation time.

Based on the enzymatic properties of coliforms, a defined substrate method was developed to overcome some limitations of the MTF and MF techniques. Unlike these methods, which eliminate the growth of non-coliform bacteria with inhibitory chemicals, the defined substrate technology is based on the principle that only the target microbes (TC and *E. coli*) are fed and no substrates are provided for other bacteria. A defined substrate is used as a vital nutrient source for the target microbe(s). During the process of substrate utilization, a chromogen or a fluorochrome is released from the defined substrate, indicating the presence of the target microorganisms.

Edberg and Edberg (1988) proposed using a combined substrate technology with the substrate ONPG for the constitutive enzyme β -galactosidase present in all coliforms and the substrate MUGlu for the specific detection of *E. coli*. The defined substrate method was basically constituted as a presence/absence test. The tubes, which are colorless after samples addition, are incubated at 35 °C. Any yellow color in the test tube (indicating the hydrolysis of ONPG) was taken as positive for TC. Any yellow tube is exposed to longwave UV light, and blue-white fluorescence demonstrates the presence of *E. coli*. No additional confirmatory tests need to be performed.

The first experiments (Edberg and Edberg, 1988) demonstrated that examination of environmental isolates of TC and *E. coli* showed sensitivity equal to that of classical methods (up to 1 CFU/100 ml) with potentially greater specificity. Data also confirmed the ability to detect injured coliforms with a maximum response time of 24 h. Rice et al. (1990, 1991) used numerous pure strains of *E. coli* to determine the detection efficiency of the defined-substrate technology with β -D-glucuronidase and showed positive results (95.5% β -D-glucuronidase-positive isolates in 24 h and 99.5% positive after 28 h of incubation). None of the non-*E. coli* isolates were positive (Rice et al., 1990).

Several commercial tests were then developed based on the defined substrate technology: Colilert (IDEXX Laboratories, Portland, ME, USA), Colisure (Millipore corporation, Bedford, MA, USA), Coli-Quick (Hach, Loveland, CO, USA). Most of these are available for a presence/absence response and for enumeration by the multi-tube technique. The most widely used among them is the Colilert test, which utilizes the defined-substrate technique with ONPG and MUGlu. Numerous and very extensive comparisons between these commercial tests and the classical MTF and MF approaches have been performed to enumerate TC and E. coli in various types of waters (Edberg et al., 1988, 1989, 1990; Clark et al., 1991; Olson et al., 1991; McCarty et al., 1992; McFeters et al., 1993; Palmer et al., 1993; Clark and El-Shaarawi, 1993; Colquhuon et al., 1995; Fricker and Fricker, 1996). The main conclusion of these studies was that these tests were effective for the detection of the coliforms from varied source waters, usually as sensitive as the MTF approach for the detection of E. coli and sometimes more sensitive for the detection of TC. More recently, the Quanti-Tray (QT) (IDEXX), which is an extended MPN version of the Colilert test (a MPN version with a limited number of tubes was also commercialized at an early stage), was compared with MF methods, specifically on drinking water samples, by Fricker et al. (1997), De Roubin et al. (1997) and Eckner (1998). They all concluded there was no significant difference for the enumeration of E. coli between the QT and MF methods, while recovery of TC was greater with the QT technique than with the MF method. McFeters et al. (1997a,b) compared the Colisure test with standard reference methods for detecting bacteria subject to chlorine stress: with Colisure, recovery of chlorine-injured TC and *E. coli* improved over standard methods, resulting in a more realistic estimate of the actual population of indicator bacteria in public water supplies.

In conclusion, tests based on the defined-substrate technology using chromogenic and fluorogenic substrates are applicable for the detection and enumeration of coliforms and *E. coli* in drinking water. These tests are easy to use and give a more rapid and more realistic estimate (especially in the presence of chlorine residual) of indicators of bacteriological contamination of waters than classical presence/absence or MTF media. These methods might be more expensive in terms of consumables than the classical methods when the latter require no additional confirmation steps. When confirmation steps are required, the costs incurred in both methods are equivalent. In all cases, enzymatic methods require less manpower and therefore their cost in terms of commercial value is lower.

4.3. MF technique conjugated to enzymatic detection of coliforms

Dahlen and Linde (1973) evaluated the incorporation of MUGlu into agar media to detect the presence of β -D-glucuronidase activity (*E. coli*). Brenner et al. (1993) developed the MI agar medium, containing the fluorogenic MUGal and the chromogenic IBDG, to simultaneously detect TC and *E. coli* in waters. The method was shown to be sensitive, selective, specific and rapid (results available in 24 h) (Brenner et al., 1996). Gaudet et al. (1996) and Ciebin et al. (1995) associated MUGlu or X-Glu with classical m-TEC and lauryl tryptose agar and compared these modified media with the classical media. The modified agar media usually showed higher or similar recovery of TC and *E. coli*.

Different commercial agar media are now available for the detection of TC and *E. coli*. They include classical agar media used for *E. coli* and coliform enumeration modified with specific chromogenic and/ or fluorogenic substrates for the detection of β -Dglucuronidase and/or β -D-galactosidase: Chromocult Coliform Agar (Merck, Darmstadt, Germany), Fluorocult *E. coli* Direct Agar (Merck) and m-ColiBlue24 broth (Hach) (Grant, 1997).

The use of these plate count methods, including chromogenic and/or fluorogenic substrates, allows far more rapid, easier and more accurate estimates of coliform and *E. coli* abundance in drinking waters than the utilization of classical media. They can be used by any laboratory able to use conventional culture methods. However, they are more expensive and they do not satisfactorily solve the problems linked to the presence of non-culturable indicator bacteria.

4.4. Direct determination of enzymatic activity by fluorimetry

The methods described above are culture-based tests and still often require 18 to 24 h to complete. More recently, the β -D-galactosidase and β -D-glucuronidase properties of TC and *E. coli* have been exploited on freshwater (Berg and Fiksdal, 1988; George et al., 2000) and seawater (Davies et al., 1995) samples in rapid assays without any cultivation steps.

George et al. (2000) finalized a protocol based on the fluorogenic substrates MUGal and MUGlu for a direct enzymatic detection of FC in freshwaters in 30 min. These methods allow a rapid and direct estimate of the level of microbiological contamination of surface water, but their detection limits (20 CFU/100 ml for FC to about 340 CFU/100 ml for TC) preclude their use for the monitoring of drinking water. An automated analyzer (Colifast CA-100 (Colifast Systems, Oslo, Norway)) has been developed on the basis of the enzymatic properties of coliforms (Ofjord et al., 1998; Berg et al., 1998). The sample is incubated (after concentration by filtration, if required) at 37 °C for TC and 44 °C for FC in a growth medium selective for coliforms and containing MUGal. If the number of coliforms in the sample is sufficient, the increase in fluorescence due to MUGal hydrolysis by the coliforms present can be measured in less than 2 h. If not, the detection of a significant fluorescence signal requires the prior growth of coliforms. The detection of 1 culturable TC takes around 11 h. This method is thus a direct determination of the enzymatic activity for samples with high coliform abundance (surface water) and involves a growth phase for samples with low coliform abundance (drinking

water). The Colifast test is more usually recommended for bathing waters, and further comparisons of analyses performed in drinking water with this analyzer and other methods are still required if conclusions on its performances are to be drawn. However, since the analysis of drinking water with the Colifast analyzer requires a growth phase, this system will be unable to detect non-culturable coliforms.

4.5. Detection of coliforms by enzymatic methods using solid-phase cytometry

Recent significant outlays has been focused on the development of rapid enzymatic procedures applicable to drinking water with the objective of reaching results within a working day's schedule. Van Poucke and Nelis (1999a) evaluated instrumental detection of fluorescent signals for decreasing the time analysis of an enzymatic membrane filtration test. They used a laser-scanning device (ScanRDI[®]-Chemunex, Ivrvsur-Seine, Paris, France) which detects and enumerates low numbers of fluorescently labeled cells by means of a solid phase cytometry technique (Brailsford, 1996). This system offers a very low quantitative direct detection limit allowing the detection of between 1 and 1000 fluorescently labeled cells distributed on a membrane. Using this system, Van Poucke and Nelis (1999b) proposed a test allowing the detection of E. coli and TC within 3.5 h, in principle also including metabolically active but nonculturable cells. The principle of the method for E. coli cells was as follows (Van Poucke and Nelis, 1999b, 2000a): E. coli present in drinking water samples are retained on a 0.4-µm pore-size filter after vacuum filtration. The trapped E. coli cells are treated with reagents to induce the enzyme β -D-glucuronidase (3 h at 37 °C) and label (0.5 h at 0 °C) the induced cells. Only the β -D-glucuronidase of viable *E. coli* can be induced and therefore only these bacteria cleave the non-fluorescent substrate (fluorescein-di-\beta-D-glucuronide), retaining the fluorescent end-product inside the cell. The fluorescence of a single cell or a microcolony (i.e. a fluorescent event) is detected by the ScanRDI[®] device: a complete laser scanning of the membrane allows enumeration of fluorescent cells in 3 min. A visual validation of fluorescent events is performed by transferring the membrane to an epifluorescence microscope fitted with a motorized stage

controlled by the ScanRDI[®] software. The procedure for detection of TC is similar, but fluorescein-di- β -Dgalactoside is used as the substrate, allowing the detection of β -D galactosidase (Van Poucke and Nelis, 2000b).

Application of the proposed methods for *E. coli* and TC to naturally contaminated and uncontaminated well water, surface water and tap water samples has indicated more than 90% *E. coli* and more than 92% TC agreement and equivalence with reference methods including mFC agar (*E. coli*), m-Endo agar (TC) and Chromocult Coliform agar (*E. coli* and TC) (Van Poucke and Nelis, 1999a,b, 2000a,b). Calculated against the latter medium, the false negative rates were 3% for *E. coli* and 3.2% for TC. In a limited number of samples, low numbers (1-3) of the target organisms were detected by laser scanning and not by conventional plate counts. If these results are confirmed in the future, this method will surely be of interest due to the short time required to analyze a water sample.

4.6. Conclusion on enzymatic methods

Addition of fluorogenic and chromogenic substrates to cultivation media (agar and liquid media) to detect the enzymatic activity of TC and *E. coli* have increased the sensitivity and the rapidity of the classical methods for estimating the microbial contamination of drinking water. Although some of these tests are able to detect injured coliforms, they do not satisfactorily solve the problem of the detection of nonculturable cells. The detection of fluorescent coliforms by solid-phase cytometry is an interesting way to decrease the amount of time required to obtain a result, but the method requires a solid phase cytometer.

5. Molecular methods

Molecular methods have been developed to increase the rapidity of analysis. They are able to achieve a high degree of sensitivity and specificity without the need for a complex cultivation and additional confirmation steps. Consequently, some of these methods permit the detection of specific culturable and/or non-culturable bacteria within hours, instead of the days required with the traditional methods. Several molecular methods applied to the specific detection of coliforms in waters and drinking waters are discussed here.

5.1. Immunological methods

Immunological methods are based on the specific recognition between antibodies and antigens and the high affinity that is characteristic of this recognition reaction. Depending on the taxonomic level of the targeted antigens, immunological methods permit detection of antigens at family, genus, species or serotype levels. Two types of antibodies can be produced: polyclonal and monoclonal antibodies, whichever are more specific to the target organism. The properties of the antigen–antibody complex can be used:

- to perform an immunocapture of cells or antigens by enzyme-linked immunosorbent assay (IMS or ELISA), or
- to detect targeted cells by immunofluorescence assay (IFA) or immuno-enzyme assay (IEA).

Over the last 10 years, attempts have been made to use immunological methods for the detection of water quality indicators in drinking water. Obst et al. (1989) developed an ELISA using a monoclonal antibody against the enterobacterial common antigen (ECA), a lipopolysaccharide which is linked within the outer membrane of Enterobacteriaceae. This method was not sensitive enough, however, and so pre-cultivation of the sample in a selective broth for 24 h was necessary in order that the detection limit of the ELISA (100,000 cells/ml) could be reached. Hübner et al. (1992) refined the ELISA with ECA monoclonal antibodies for the routine detection of Enterobacteriaceae and showed a degree of similarity of 98% with the standard TC method (multiple-tube fermentation). Levasseur et al. (1992) exhaustively analyzed the specificity of ECA monoclonal antibodies after screening 259 Enterobacteriaceae strains and 125 other Gram-negative bacteria. They found cross-reactivity with, among others, different strains of Aeromonas, which are important competitors to coliform bacteria growing on m-Endo media. Same false positive reactions were reported by Hübner et al. (1992), who mention cross-reactivity with several Gram-negative (Pseudomonas and Aeromomas) and Gram-positive (*Bacillus*) bacteria, when ECA monoclonal antibodies are used for Enterobacteriaceae detection.

The ELISA is a rapid, simple and quite sensitive test, which allows the detection of less than 10^{-9} g of antigenic-protein (Stryer, 1988). However, assay limitations are often associated with the specificity of the antibody used, the concentration of both antibody and antigen and the type of reaction solution used. In addition, the solid matrix often leads to non-specific binding of the antigen or of the second antibody (Kfir and Genthe, 1993). This method is very useful for testing monoclonal antibodies or detecting spiked microorganisms. Nevertheless, its application to the detection of specific cells from a natural contaminated sample is limited (Hanai et al., 1997). High levels of non-targeted microflora and divers materials associated with the sample may interfere on the level of the specificity of the ELISA method.

Immunofluorescence assay allows the identification and enumeration of a single specific cell in a natural sample (Campbell, 1993). Assays can be performed by a direct or an indirect procedure. In a direct immunofluorescence procedure, the specific antibody is directly conjugated with a fluorochrome. An indirect procedure involves the binding of the specific primary antibody to the targeted antigen followed by the addition of a fluorochrome-labeled antibody directed against the first antibody. The advantage of this procedure is that the secondary antibodies can easily be obtained from a commercial supplier with a range of conjugated fluorochromes (Hoff, 1993). Enumeration of fluorescently labeled cells can rapidly be achieved by using epifluorescence microscopy or solid-phase cytometry after filtration of the water sample, or by flow cytometry. Zaccone et al. (1995) applied a direct immunofluorescent assay on membrane for the evaluation of fecal pollution in samples from coastal and lake waters. They compared the FC recovery efficiencies of IFA achieved by epifluorescence microscopy and MF methods and showed higher IFA than CFU counts by 2 orders of magnitude. Nevertheless, they reported the existence of a threshold below which the IFA method cannot be applied (when specific bacteria were below 1% of the total population) and mainly related to the particles content interference, when large volumes were filtered.

While IFA allows specific identification and detection at a single-cell level, it does not provide information on the physiological status of cells, or on whether they are living or dead. An innovative immunofluorescence assay was developed by Rockabrand et al. (1999), which determines the physiological status of a cell by profiling stage-specific growth proteins. Differentiation of coliform growth stages was based on the presence of three cytosolic proteins: DnaK, a metabolically stable protein; Dps, an important protein in stationary-phase or dormant cells which is inversely correlated with growth rate; and Fis, which plays a critical role in coordinating rRNA synthesis with growth. Quantification of the cytosolic proteins was performed by using enterobacteria-specific fluorochrome-labeled monoclonal antibodies (anti-DnaK, anti-Dps and anti-Fis). The specificity of the antibody probes was tested by comparing the results with those obtained by a 16S rRNA probe specific to Enterobacteriaceae (Mittelman et al., 1997) on pure cultures of six Enterobacteriaceae and one other member of the gamma subdivision of the Proteobacteria subclass. Simultaneous probing of wastewater samples with the DnaK antibody and the 16S rRNA oligonucleotides showed that both methods gave similar results. To our knowledge, these specific antibodies have not been applied to the detection of coliforms in drinking water.

The major limitation of immunological methods applied to drinking water quality monitoring is linked to the very low number of targeted cells in the samples. Analyses performed on large volumes of filtered water samples are limited, depending on the particles content (Faude and Hofle, 1997; Hanai et al., 1997). Nevertheless, enumeration of diluted specific cells can be obtained by means of immunomagnetic separation (IMS, Dynal). This method uses magnetic beads coated with monoclonal or polyclonal antibodies, making it possible to reduce the sample-associated background by purifying and concentrating targeted cells. Although the IMS method has been widely applied to the detection of low numbers of cells from food samples (Peng and Shelef, 1999; Mansfield and Forsythe, 2000), few studies reported how it performs on drinking water samples. Recently, Pyle et al. (1999) proposed a combination of IMS and solidphase laser cytometry for a sensitive detection of E. coli O157:H7 spiked in water. Purification and concentration steps were realized using supermagnetic beads coated with anti-O157 rabbit serum and a

magnetic separation, making it possible to remove cells fixed on beads from the sample. They performed various analyses on the removed cells, such as enumeration of culturable cells and respiring cells. Culturable cells were enumerated by MF and identified by a direct IFA method in combination with ScanRDI[®] detection. This polyphasic approach applied on spiked water samples showed a higher level of sensitivity than cultivation-based method.

The use of immunological methods for the detection of specific microorganisms is a rapid and simple technique, the accuracy of which mainly depends on the specificity of the antibody (Kfir and Genthe, 1993). One way to increase specificity is to select monoclonal antibodies which are highly specific in their action against a specific epitope. However, since an epitope can be present in more than one antigenic agent, a rigorous specificity testing of the monoclonal antibodies synthesized with closely and distantly related bacterial strains must precede the routine testing of environmental samples. Most likely due to this problem of cross-reactivity with other bacterial strains, the use of immunological methods for the detection of coliforms and E. coli has not yet been successful. Furthermore, at our knowledge, real polyvalent antibodies targeted against all environmental strains of E. coli do not exist at this time. The application of monoclonal antibodies to the detection of specific organisms in a natural environment, particularly of coliforms in drinking water, probably needs more complex investigations directed towards antibodies specificity and bacterial abundance in a drinking water sample. Since monoclonal antibodies have approximately the same specificity as the 16S rRNA oligonucleotide probes and since their production is much more laborious, there is no advantage to use immunological methods over the fluorescent in situ hybridization (FISH) method (see next section). One advantage over FISH would be that the use of an antibody like anti-Fis (Rockabrand et al., 1999) would differentiate the physiological status of the coliforms detected in the water sample.

5.2. Nucleic acid-based methods

Most of the nucleic acid methods use molecular hybridization properties, which involve the complementary sequence recognition between a nucleic probe and a nucleic target. A hybridization reaction can be realized between a nucleic DNA probe and a chromosomic DNA sequence (DNA-DNA hybridization) or an rRNA or tRNA sequence (DNA-RNA hybridization). Specificity, here, depends on the phylogenetic degree of conservation of the target within the taxonomic target group. These methods offer taxonomic information at different levels, such as classes, genera, species or subspecies. Some of them can be performed without the need for a complex cultivation step, thereby permitting the detection of specific bacteria within hours, instead of few days required with the cultivation-based methods. The more frequently used nucleic-acid-based methods are the polymerase chain reaction (PCR) and the in situ hybridization (ISH) methods. Assessment of their performance in the detection of coliforms in drinking water samples is discussed below.

5.2.1. Polymerase chain reaction methods

PCR allows a DNA target fragment to be amplified by cycling replication. This replication, which can be performed in vitro or in situ, consists of a chain reaction catalyzed by a DNA polymerase (*Taq* polymerase) and the use of oligonucleotidic primers. Detection specificity depends on the degree of homology and complementarity between target and primer and on hybridization temperature. The cycling of PCR results in an exponential amplification of the amount of the target sequence and significantly increases the probability of detecting a rare sequence or relatively low numbers of target microorganisms in a sample (Bej et al., 1990; Waage et al., 1999a,b; Burtscher et al., 1999).

The most commonly used PCR method applied to the detection and identification of bacteria includes an in vitro cycling replication after a DNA extraction step. Amplification is performed on the nucleic acid content obtained by a cellular lysis followed by a chemical extraction. These extraction steps can be performed on bacterial cells retained on a membrane filter (Bej et al., 1991a; Juck et al., 1996; Iqbal et al., 1997; Tsen et al., 1998). The PCR amplification process involves: (i) a DNA denaturation from double- to single-stranded DNA, (ii) annealing primers to the single-stranded DNA at a specific hybridization temperature, and (iii) primer extension by a DNA *Taq* polymerase. Amplification of a sequence by PCR typically requires 20 to 40 cycles. PCR products are detected after electrophoresis on agarose gel and after staining of amplification products by a fluorochrome dye or by hybridization with a labeled probe.

The PCR method has often been described for the detection and identification of microorganisms in foods, soils, sediments and waters. If detailed protocols for PCR utilization on real environmental samples were available for several years (Trevors and van Elsas, 1995), application of this method to the detection of microorganisms in drinking water is recent. Several protocols have been developed for detection of TC, FC (*E. coli* and related microorganisms) and pathogens including *Salmonella* spp. (Table 2).

For coliforms, primers development has been difficult since the coliform group, as defined by the water industry, is a diverse group containing many genera and excludes some which are closely linked. As a consequence, primers must be specific enough that they do not detect closely phylogenetically related non-coliforms. Primers based on the lacZ gene have been used for the detection of coliforms because conventional coliform monitoring methods are based on the expression product (β -galactosidase) of this gene (Bej et al., 1990, 1991a,b; Fricker and Fricker, 1994). PCR product of different sizes (326 and 264 bp) were created using different primer sets and resulted in the detection of TC in amounts as small as 1 cell/100 ml using radiolabeled gene probes (Bej et al., 1990, 1991a). Fricker and Fricker (1994) investigated the specificity of the same primers set as Bej et al. (1991a) on 324 cultured coliform strains. They concluded that another set of primers should be developed since the one based on the lacZ gene did not allow the discrimination of some Hafnia alvei and Serratia odorifera strains.

For the specific detection of *E. coli*, a region of the *malB* gene which codes for a maltose transport protein was the first target sequence proposed (Bej et al., 1990). This region includes the *lamB* gene which encodes a surface protein recognized by an *E. coli*-specific bacteriophage. However, members of the *Shigella* and *Salmonella* genera could also be detected using this primer set. The use of the *uidA* gene was then proposed for the detection of *E. coli* (Bej et al., 1991a,b; Tsai et al., 1993). Both the regulatory region (*uidR*) and the *uidA* gene encoding the β -D-glucuronidase (GUD) enzyme were used subsequently with

Table 2							
Use of PCR	amplification	for the	detection	of microor	ganisms	in	water

i		0			
Type of bacteria and references	Type of strains	Primer set	Amplified region ^a	Detection	Detection limit
Total coliforms					
Bej et al., 1990	culture strains	ZL-1675; ZR2025	<i>lacZ</i> (326 bp)	electrophoresis radiolabeled	1 to 5 cfu
Bej et al., 1991a,b	culture strains	LZL-389; LZR653	<i>lacZ</i> (264 bp)	electrophoresis radiolabeled	1 cell (by multiplex PCR)
Fricker and Fricker, 1994	culture strains	LZL-389; LZR653	<i>lacZ</i> (264 bp)	electrophoresis	_
E. coli, Salmonella spp., Shigell	a spp.				
Bej et al., 1990	culture strains	BL-4910; BR-5219	<i>lamB</i> (309 bp)	electrophoresis radiolabeled probes	1 to 5 cfu
E. coli, Shigella spp.					
Bej et al., 1991a,b	culture strains	UAL-1939; UAR-2105;	uidA (147 bp),	electrophoresis radiolabeled	1 cell (by multiplex PCR)
		URL-301; URR-432	uidR (154 bp)	probes	
Spierings et al., 1993	culture strains	EC5; EC8c ₂	<i>phoE</i> (348 bp)	electrophoresis radiolabeled probes	_
Fricker and Fricker, 1994	culture strains	URL-301; URR-432	<i>uidR</i> (154 bp)	electrophoresis	-
Juck et al., 1996	culture strains	uidA 858; uidA1343;	uidA (486 bp),	electrophoresis	2-20 cells/100 ml by nested PCR
		uidA 1047; uidA 1232	uidA (186 bp)		
Iqbal et al., 1997	culture strains and polluted	UAL-1939; UAR-2105:	uidA (147 bp),	electrophoresis radiolabeled probes	-
	river water samples	URL-301; URR-432	uidR (154 bp)	ī	
Tsen et al., 1998	culture strains	16E1, 16E2; 16E3	V_3 and V_6 regions of 16S rRNA (584 bp)	electrophoresis nonisotopic probes	1-2 cells/100 ml
Salmonella					
Waage et al., 1999a	environmental and clinical isolates	SAL-1F; SAL-2R; SAL-3F; SAL-4R	ST14 (438 bp), ST11 (312 bp)	electrophoresis	2 cfu by nested PCR

^a Amplified regions details: lacZ gene: encodes for the enzyme β -galactosidase; lamB gene: encodes for a surface protein that is recognized specifically by the *E. coli* bacteriophage lambda; *uidA* gene: encodes for the enzyme β -D-glucuronidase; *uidR* gene: encodes for the regulatory region of *uidA*; *phoE* gene: encodes for a pore-forming outer membrane protein.

success (Fricker and Fricker, 1994; Juck et al., 1996; Iqbal et al., 1997). The primer sets are specific for *E. coli* and *Shigella* spp. Bej et al. (1991b) reported better sensitivity of the PCR method compared to the MUGbased defined substrate tests. Many MUG-negative *E. coli* strains, which include the pathogenic *E. coli* of serotype O157:H7, were detected after PCR amplification of the *uidA* gene. Moreover, Feng et al. (1991) have shown that many MUG-negative strains possess the *uidA* gene even if the gene product (the enzyme β -D-glucuronidase) is not expressed. Other primer sets designed for two different regions have been proposed for the detection of *E. coli*, one of them coding for an outer-membrane protein (*phoE* gene) (Spierings et al., 1993) and the other coding DNA sequences for the V3 and V6 regions of the 16S rRNA genes of pathogenic and non-pathogenic strains of *E. coli* (Tsen et al., 1998). These primer sets permit the specific detection of *E. coli*, but also of *Shigella* species when the suggested sequences are amplified.

To increase the specificity of the detection by PCR, some authors have proposed the use of multiplex PCR, which consists of simultaneously amplifying different DNA fragments. Bej et al. (1991b) modified this approach to detect gene sequences related to the TC group and those associated with enteric pathogens including E. coli, Salmonella spp. and Shigella spp. Multiplex PCR may not perform well with all primer blends, however. The composition and length of primer oligonucleotide, as well as the size of the amplified fragments, may influence each PCR amplification. For example, multiplex amplification of TC and E. coli is performed using equimolar concentrations of both primers, while non-equimolar primer concentrations are necessary for the simultaneous detection of Legionella pneumonia and all bacteria of the genus (Bej et al., 1991a).

To detect low levels of pathogens or indicator microorganisms in water samples, a concentration step such as filtration must first be performed. Bej et al. (1991a) reported using filtration methods and releasing the DNA by freeze-thaw cycling without affecting the PCR amplification. When visualization of PCR products is performed using hybridization with radioactive probes, this method permitted a detection limit to up to 1 cell/100 ml. However, applying this method to routine monitoring could be difficult since visualization of the product by hybridization with a radioactive probe may require 2 to 3 days of exposure. Non-radioactive detection systems, like biotinylated primers, may be a useful substitute for more rapid detection of target DNA-probe hybrids. Juck et al. (1996) developed a protocol for the rapid detection of low concentrations of E. coli in water samples based on the nested PCR protocol and a modification of the filtration protocol proposed by Bej et al. (1991a). Nested PCR involves two consecutive rounds of PCR amplification. The first run is performed as usual, with primers annealed to specific target regions (templates) followed by primers extension. The second round uses the product of the first amplification as the template for another round of annealing and extension with different primers. Usually the product of the second amplification results in

a fragment that is internal to the product of the first round of PCR amplification. Nested PCR increases amplification efficiency since sub-detectable levels of PCR products generated during the first round are amplified to reach detectable levels in the second round. The use of nested primers provides an additional level of specificity since the second PCR round can only be performed if the correct sequence (complementary to the inner primers) was amplified during the first round. Nested PCR protocols were used for the detection of E. coli (Juck et al., 1996) and some pathogens (Delabre et al., 1997) in drinking water, whereas Waage et al. (1999a,b) applied them to the detection of low numbers of Salmonella spp. and Y. enterocolitica cells in waters. This technique permits a more rapid detection (6 to 8 h) than the usual PCR (few days), since confirmation of the correct sequence amplification by probe hybridization is no longer necessary.

Despite its sensitivity, it is difficult to use PCR for the quantification of microorganisms. Two techniques have mainly been developed for DNA quantification by PCR: the most-probable-number PCR (Toranzos et al., 1993) and the competitive PCR (addition of a DNA competitor for the PCR stage to calibrate the PCR efficiency) (Zachar et al., 1993). To our knowledge, these techniques have not been applied up to now for the quantification of coliforms in drinking water. Thus, at present, it does not seem possible to quantify coliform density in drinking water using PCR. Up to now, the MPN-PCR remains inaccurate and competitive PCR requires considerable preparation before a good DNA competitor is applied to the water samples.

Another promising approach is real-time quantitative PCR, which consists in monitoring the fluorescently PCR products as they are amplified (Heid et al., 1996). The kinetics of PCR products accumulation is an exponential phase followed by a plateau phase. Occurrence of this plateau depends on several uncontrolled parameters, such as the dimerization of primers and the appearance of inhibitors. Real-time quantification is more reliable than end-point quantification because measurements are carried out during the exponential phase. It is also possible to detect inhibition problems of the PCR reaction due to inhibitors co-purified with DNA. This approach has recently been applied to the detection of enterohemorhagic and enterotoxigenic *E. coli* strains in clinical microbiology (Bellin et al., 2001; Carroll et al., 2001). When amplified PCR products are stained with SYBR Green dye, using a LightCycler (Roche, Mannheim, Germany) or a GeneAmp (Applied Biosystems, Foster City, USA), the real-time PCR method showed greater rapidity and higher sensitivity and specificity in comparison to the duplex PCR assay with traditional gel analysis (Carroll et al., 2001). Nevertheless, this approach has not yet been widely applied to specific detection of microorganisms present at low concentration in environmental samples, but is likely to be developed in the future.

More recently, Tani et al. (1998) have proposed a direct enumeration of targeted cells using a direct in situ PCR assay. The amplification of a specific sequence is performed from a single-copy gene present in the cell. The usual PCR protocol must be modified so that nucleic acid sequences can be amplified in vivo. With proper fixation and permeabilization conditions, the oligonucleotide primers and other reaction components are able to diffuse into the cells, and, upon thermal cycling, amplify specific target sequences. PCR products are labeled by digoxigenin-UTP (DIGdUTP), and anti-DIG Fab' fragments conjugated with fluorescent dye are used for detection by epifluorescence microscopy. This approach allows a direct visualization of the fluorescent amplification products at a single-cell level and consequently a possible direct enumeration of labeled-cells. These authors have applied this new PCR method to the direct enumeration of E. coli from freshwater sample. Results showed a weak fluorescence intensity signal of targeted cells. Nevertheless, they concluded that image analysis allows a direct enumeration of E. coli target cells. Even though the in situ PCR protocol seems promising, it has not been used for the routine detection and enumeration of microorganisms in water.

Many restrictions associated with the PCR amplification of DNA need to be resolved before this method can be used for the sensitive analysis of coliforms in drinking water samples. Even though this method has been used with some success for the detection of coliforms and *E. coli*, it should be pointed out that most of these studies were performed on water samples spiked with cultured strains of bacteria (Table 2) and that validation was rarely, or not, carried out on environmental samples. Despite the fact that PCR is recognized as a highly specific detection and identification method, it can present some limitations when applied to the detection of those cells in a natural sample that are viable or metabolically active without being culturable. Indeed, PCR amplification applies to nucleic acid extracted from viable and culturable, viable and non-culturable or dead cells. Consequently, PCR-based assays cannot provide information on the physiological status of targeted cells. Another limitation inherent to PCR analysis of environmental samples is the frequent inhibition of the enzymatic reaction: humic substances are known as polymerization enzyme (Taq-polymerase) inhibitors, and colloid matter has a high affinity for DNA (Way et al., 1993). The presence of these elements in a water sample can therefore considerably decrease the amplification yield of PCR applied to the detection of greatly diluted bacteria. They can also limit the revealing of the amplification products (Straub et al., 1995).

5.2.2. In situ hybridization techniques

ISH uses oligonucleotide probes to detect complementary nucleic acids sequences. This method exploits the ability of nucleic acids to anneal to one another in a very specific complementary way to form hybrids. The probes are specific because they are built from, and are complementary to, selected nucleic acids sequences which are unique to a given microorganism, species or group. The probes can target either DNA or RNA molecules. Use of rRNA sequences (5S, 16S and 23S) to study phylogenetic relationships on the basis of their divergence and to develop determinative hybridization probes is now well established (Amann et al., 1995). The sequencing of more than 2500 of the bacterial species 16S rRNA currently confers on these genes a very high informative value at a phylogenetic level. Sequence comparisons make it possible to define targeted regions which are perfectly conserved within different taxonomic levels and consequently specific to these levels, from domains to subspecies. Also of interest in targeting rRNA molecules is the high level of rRNA molecules copies, which is in turn linked to the large number of ribosomes per cell. The number of ribosomes varies, generally between 10³ and 10⁵ per bacteria, according to the species and to the physiological state of the cells, and is directly correlated with the cellular growth rate (Amann et al., 1995). Probes

specific to rRNA (mainly 16S and 23S rRNA sequences) have become the standard tools for organism identification (Olsen et al., 1986).

Several oligonucleotide probes are commercially available and the choice of whether to use them or design new, specific ones depends largely on the application. To find the continuous target sequence unique to a specific microorganism, researchers rely on computer-aided sequence comparison available in the Ribosomal Database Project (RDP) (Maidak et al., 1996), in GenBank for DNA (Benson et al., 1999), or in the ARB software package (Max Planck Institute, Bremen, Germany). For better specificity, the target sequence should be short (15 to 30 bases) and have at least two to three different nucleotides with homologous sequences of closely related organisms (DeLong, 1993). Early work on in situ hybridization relied on radioactive probes to reveal and detect the probetarget hybrid (Olsen et al., 1986). Current work on rRNA in situ hybridization uses fluorescent-labeled nucleotide probes almost exclusively to detect hybridization (FISH). The popularity of the FISH technique is due to its advantages over radioactive labeling, which include sensitivity, speed of visualization of single cells (by means of microscopy or cytometrical devices), stability of the hybridization products, safety, diminished detection time, multiple labels (multiple colors) and ease of use (Richardson et al., 1991; DeLong, 1993; Swinger and Tucker, 1996). Fluorescein and rhodamine dyes are the most frequently used fluorochromes (DeLong et al., 1989; Amann et al., 1990; Manz et al., 1992; 1993; Wagner et al., 1994), but the CY3 dye, brighter and leading to lower non-specific fluorescence than the other fluorochromes, has recently been attracting more attention (Wessendorf and Brelje, 1992; Glockner et al., 1996; Zarda et al., 1997; Kalmbach et al., 1997a; Ouverney and Fuhrman, 1999). In practice, FISH procedure steps are: cell fixation, hybridization (specificity and stringency depend on hybridization temperature and time, salt concentration, probe concentration and length), post-hybridization washing (to remove unbound or non-specifically bound material) and detection. Hybridized cells are usually detected by epifluorescence microscopy and a counterstain such as DAPI (4',6'-diamidino-2-phenylindole) or orange acridine is used to determine the total number of cells. Depending on the concentration of targeted cells in

the sample and to increase resolution, FISH detection can be performed by means of flow or solid-phase cytometry. Flow cytometry enables quantification of the fluorescence intensities for each target-probe hybrid (Fuchs et al., 1998).

For TC, the development of a specific 16S rRNA probe for this group is not possible, since the coliform group as defined by the water industry is a group containing bacteria from genera that are phylogenetically different. As a result, probes (Table 3) were developed for the Enterobacteriaceae family rather than for TC. The first, ENTERO (Mittelman et al., 1997) was developed for clinical detection of urinary tract infections. The second, ENT1 (Loge et al., 1999) was developed for application on wastewater samples. These two probes are different in their composition and in the hybridization conditions. The ENTERO probe is longer-25 bases with a C+G content of 48% compared to 17 bases and a 70% C+G for the ENT1 probe. Research within the RDP (Maidak et al., 1996) showed higher specificity for the ENT1 probe for Enterobacteriaceae species than the ENTERO probe (Rompré and Baudart, NSERC Industrial Chair on Drinking Water, Ecole Polytechnique Montreal, personal communication).

Sequences of rRNA target probes for the detection of E. coli have also been published. The EC1531 probe (Poulsen et al., 1994), complementary to a 23S rRNA sequence, is composed of 20 nucleotides with a C+G content of 55%. Shi et al. (1999) used this probe for the detection of E. coli after modifying the hybridization conditions, but no results were mentioned. The probe had only been used as a control in an experiment using a microcosm extract spiked with E. coli DNA. More recently, Regnault et al. (2000) developed the Colinsitu probe for the detection of E. coli and E. fergusonii in urine, water (rivers and sewage) and food samples. The Colinsitu probe complements a 16S rRNA sequence and is composed of 24 nucleotides (C+G content of 46%). This probe showed a good specificity for the visualization of E. coli from the various media tested and has newly been used with success for the detection of E. coli in drinking water samples (Delabre et al., 2001).

The FISH technique appears to be a highly specific detection method at a cellular level; however, it may have some limitations when applied to the detection of nutrient starved bacterial cells disseminated in drink-

	$\mathbf{D}_{\mathbf{r}}$	"DNIA 4 4	Q	References	
Probe	Probe sequence $(5-3)$	fRNA target position	Specificity		
ENTERO ^a	CATGAATCACAAAGTGGTAAGCGCC	16S, 1458-1482	Enterobacteriaceae	Mittelman et al., 1997	
ENT1	CCGCTTGCTCTCGCGAG	16S, 1273-1289	Enterobacteriaceae	Loge et al., 1999	
EC1531	CACCGTAGTGCTCGTCATCA	238, 1531-1550	E. coli	Poulsen et al., 1994	
COLINSINTU	GAGACTCAAGATTGCCAGTATCAG	16S, 637-660	E. coli	Regnault et al., 2000	
PNA probe	GCAAAGCAGCAAGCTC	16S, 71–86	E. coli	Prescott and Fricker, 1999	

 Table 3

 Oligonucleotide probes used for identification of *Enterobacteriaceae* and *E. coli* in the environment

^a Named here ENTERO for easier differentiation since no name was given by the author.

ing waters. These limitations are linked to a low bacterial ribosome content and thus to the small number of 16S rRNA targets of these cells, which induces weak fluorescent hybridization signals (Amann et al., 1995; Lebaron et al., 1997). This is probably why FISH is rarely described in the drinking water literature at this time. As a consequence, fluorescence amplification systems, such as multiple probing (instead of monolabeled probing in the conventional FISH technique), interactive properties of the biotine-avidine complex and/or horseradish peroxydase conjugated with fluorochrome-tyramidesubstratum, could represent a way to increase the intensity of the fluorescent signal given off by starved hybridized cells (Lebaron et al., 1997; Schönhuber et al., 1997). Recently, Prescott and Fricker (1999) reported the use of a peptide nucleic acid (PNA) probe for in situ hybridization and detection of E. coli in tap water. The hybridization results were similar to those yielded by the plate count method. A PNA is a synthetic nucleic acid in which the sugar backbone is replaced with a peptide backbone. The advantages to using PNA instead of DNA probes include better resistance to nuclease attack, hybridization independent of salt concentration, more specific binding and shorter probes sequences, to achieve greater sensitivity. Since the PNA probe binds more strongly to the target site, hybridization reaction can be completed in a shorter time (30 min). To our knowledge, except for the E. coli PNA probe (Prescott and Fricker, 1999), none of these probes has been used for coliforms or pathogenic bacteria monitoring in drinking water. Before they are accepted as a sensitive coliform enumeration method, they must first be subjected to a validation stage so that the probe most suitable for the samples analyzed can be chosen and to optimize the hybridization conditions if necessary.

Cell viability is a major question arising in the utilization of FISH for monitoring contamination of drinking water. It is established that the rRNA content of the bacterial cells is correlated directly with the growth rate (Amann et al., 1995). However, the rRNA content of a bacteria may not completely reflect its physiological status. It appears that a small number of rRNA molecules can remain for a relatively long period after the loss of culturability. S. aureus and E. coli rRNA were still detected 48 h after moderate heat activation or UV irradiation (McKillip et al., 1998). Sheridan et al. (1998) still found E. coli 16S rRNA 16 h after thermal inactivation at 100, 80 and 60 °C. Finally, Prescott and Fricker (1999) exposed an E. coli strain to 1.5 mg/l of chlorine for up to 30 min. Using the PNA probe and the tyramide fluorescent amplification system (TSA kit; Perkin Elmer Life Science, Ontario, Canada), they still detected bacteria immediately after chlorination, while no cells were detected by the plate counts or Colilert methods. Two weeks after chlorination, 10% of the cells were still being detected by the PNA probe-TSA kit. This can be viewed as an advantage to the method in that non-culturable coliforms can be detected, or as a disadvantage in that dead cells might be enumerated. Further investigations on the physiological state of the bacteria enumerated by FISH is still required to come to a conclusion on this question. One possible way to study the issue is to couple the direct viable count technique (DVC) (Kogure et al., 1979) with FISH detection (Nishimura et al., 1993; Kalmbach et al., 1997b). This coupling was very recently applied to E. coli detection in drinking water by Delabre et al. (2001). These authors found significantly higher E. coli abundance with their protocol than by plate counts.

FISH is currently considered as a highly specific cellular detection method, and as relatively easy to perform. The specificity of detection depends on the specificity of the oligonucleotidic probe and on the stringency of hybridization conditions used. Identification of the target sequence remains tedious, however, and FISH cannot be applied to the detection of nonphylogenetically identified micro-organisms, such as coliforms. In this case, work can be done on Enterobacteriaceae, the nearest phylogenetically identified group that can be considered as a potential indicator of fecal contamination.

6. Conclusions

The purposes of this work were as follows:

- to compile an inventory of traditional and more recent coliform group detection methods, mainly those applied to the analysis of the microbiological quality of drinking water, and
- to evaluate the limitations of these methods in terms of sensitivity and application.

Today, the MF technique is the method most widely used for the enumeration of coliforms in drinking water. This technique, simple to perform and inexpensive, requires at least an overnight incubation period and a confirmation test (24 to 72 additional hours) after the initial typical colony investigation. Furthermore, when standard agar media are used with this technique is not possible to recover stressed or injured coliforms. Specially developed media (such as m-T7) and the addition of specific compounds (such as pyruvate) improve the recovery rate of these stressed or injured cells. Moreover, a long response time, the length of which depends on the biochemical test used for the confirmation step, are still limiting factors. The important challenges for the development of new coliform detection methods are to improve the specificity of the method, which could eliminate the time-consuming confirmation step, to take into account stressed and injured cells and to reduce the analysis time.

A specific enzymatic activity investigation should improve the sensitivity of coliforms and *E. coli* detection, using β -D galactosidase and β -D glucuronidase. Many chromogenic and fluorogenic substrates exist for these enzymatical detection activities, and various commercial tests based on these substrates are available. Numerous comparisons between these tests and the standard methods have shown that they may be a suitable alternative to the MF technique. The tests are easy to perform, require only basic laboratory equipment, and show high specificity and sensitivity. They are, however, more expensive, even more so if the incubation time is reduced (to about 18 h), and they take too long for same-day results.

Detection of coliforms by means of the enzymatic approaches at a single-cell level in combination with fluorescent probes can also be proposed, as these methods permit very specific and rapid detection without the need for a cultivation step. An acceptable alternative to the conventional culture approach is the rapid, real-time detection method developed by Van Poucke and Nelis (2000a,b). This method is based on the direct screening, with a solid-phase cytometer, of fluorescent products of the β -D-glucuronidase (*E. coli*) or of the β -D-galactosidase (total coliforms) activities at a cellular level. This new approach allows to increase the sensitivity level while being much more rapid than other commercial tests.

Immunological methods, which have been very popular, are easy to apply but still have some limitations linked to cross-reaction with non-targeted cells of commercialized antibodies. PCR methods offer a higher level of specificity detection. Nevertheless, they have major limitations when applied to natural samples, including low amplification rates linked to the presence of inhibitor substances and the lack of information on the physiological activity of cells. The PCR method requires highly skilled staff as well as dedicated laboratory space and specific reagents to avoid contamination of the samples by external DNA, particularly when nested-PCR is required. Furthermore, this method was developed for identification proposes, not for quantification. Quantification with PCR has been proposed, but these techniques are either not precise or necessitate extensive preparation.

The FISH method should also be considered as an alternative enumeration method for the investigation of coliforms in drinking water, as it provides quantitative data within one day. Currently, the use of oligonucleotide probes for the enumeration of coliforms is not widespread. The major problem with applying FISH to the enumeration of fecal index cells in drinking water samples is linked to the physiological status of drinking water bacteria (starvation or stress due to disinfectant). Actually, these bacterial cells are usually characterized by small ribosomal content, complicating the analysis of positive fluorescent signals. The use of PNA instead of DNA probes and/or the use of fluorescence amplification systems should provide enhanced sensitivity and fluorescence intensity of positive signals. The application of FISH to drinking water samples currently requires more development in order to achieve optimal enumeration of starved or stressed indicator cells.

Usually, coliforms are found in small numbers in drinking water samples. Consequently, investigations at the cellular level of diluted targeted cells involves a strategic choice of analytical instrument. Recently, Joux and Lebaron (2000) analyzed the quantitative and qualitative limitations of various fluorescence analysis tools (epifluorescent microscope, flow cytometer, confocal scanning laser microscope and the new solid-phase laser scanning cytometer). Due to its ability to detect a single cells on a 25-mm diameter membrane, semi-automatic detection of positive fluorescent signals by means of solid-phase cytometer should offer acceptable sensitivity for the direct enumeration of diluted targeted cells.

The emergence of new detection and real-time methods is linked to the need for a better assessment diagnostics for the microbiological quality of water. This objective can be reached through an increase in detection specificity and a reduction in analysis time. Several methods offer direct information at the cellular level through taxonomical and/or physiological investigation. Some of them permit, notably in stressful environments, an increase in detection levels and higher enumerations than standard culturability-based methods. By eliminating the time-consuming confirmation step, they also allow a reduction in analysis time and thus a quicker response regarding health related problems. These methods are still in the development stage, however. Technical complexity and the correspondingly high associated costs limit currently their potential for becoming standardized methods for the detection of coliforms in drinking water samples.

Furthermore, to our knowledge no in situ work, except the Delabre et al. (2001) study, has yet demonstrated that a significant proportion of the cell indicators are found in a viable or active but nonculturable states in distribution systems. If this is the case, the next research step will be to evaluate the potential impact of these viable or active but nonculturable cells in terms of pathogenicity, but also in terms of regrowth potential.

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