THE MICROBIOLOGICAL QUALITY OF WATER

Edited by

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Published by the Freshwater Biological Association

Invited papers from a specialised conference held in London
on 12-13 December 1995
by the
Freshwater Biological Association,
The Ferry House, Far Sawrey, Ambleside, Cumbria LA22 0LP
and
International Water Supply Association,
1 Queen Anne's Gate, London SW1H 9BT, UK

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ISBN 0-900386-57-6

Cultural methods of detection for microorganisms: recent advances and successes

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Most microbiological methods require culture to allow organisms to recover or to selectively increase, and target organisms are identified by growth on specific agar media. Many cultural methods take several days to complete and even then the results require confirmation. Alternative techniques include the use of chromogenic and fluorogenic substances to identify bacteria as they are growing, selective capture using antibodies after short periods of growth, molecular techniques, and direct staining with or without flow cytometry for enumeration and identification. Future microbiologists may not use culture but depend on the use of specific probes and sophisticated detection systems.

Introduction

In the beginning there was the Most Probable Number (MPN) or multiple tube technique for the isolation of coliforms and *Escherichia coli* from water. The test was based upon a nutrient medium (MacConkey Broth) containing lactose and bile salts as an enrichment medium (Anon 1956). This was replaced at a later date by the introduction of the chemically-defined medium, Minerals Modified Glutamate Medium (PHLS 1969), as the standard for water testing. Both media detected bacteria by the fermentation of lactose in the presence of a surfactant, with the production of acid and gas. Incubation was at 37°C for up to 48 h. A lack of total specificity in the medium necessitated the requirement of subculture and confirmation, and this took a further 48 h. Hence a confirmed result took 4 days and this has always been seen as unsatisfactory. In addition the test was labour intensive and open to interpretation by, in particular, the failure to produce gas. The final result was based on the analysis of 100 ml of water.

In 1953, Windle-Taylor *et al.* described a membrane filtration test for the analysis of drinking water and by the mid-1970s this had gained wide acceptance. The isolation medium was still based on surfactant and lactose but now typical colonies could be identified and counted. Membranes could be read in as little as 14 h although a presumptive result took 18 h to obtain. The medium still lacked specificity and confirmation taking a further 24 h was still required. Nevertheless, results became available at the beginning of the next working day. The method required less preparation but was still labour intensive. It was also open to interpretation. Colonies which failed to ferment lactose, even though they might be indicators, were not counted, and any which failed to confirm by acid, gas or indole production were discarded. Newly developed biochemical testing kits designed for the medical microbiologist were of some help in sorting out problems, although they were not defined as part of the testing procedure. We still manage to confirm the occasional strain of *Yersinia pestis*. Membrane filtration is based on the analysis of 200 ml of water, albeit for two separately identified tests.

Plate counts (Anon 1994) are a standard method for detecting microorganisms in water. These rely on the growth of bacteria in a nutritionally rich culture medium. Such bacteria are under stress from being in nutritionally deplete water and may be additionally damaged through water treatment or disinfection. It has long been recognised that cultural methods only detect 0.01 to 1% of the total bacterial population present in any water sample.

In-situ testing

Colonies that grow on selective media contain a wide range of enzymes which can be utilised for the purpose of biochemical confirmation. Dufour & Cabelli (1975) described an *"in-situ"* test procedure for differentiating coliforms within the coliform group. These tests were based on urease, oxidase and indole, all conducted on the colony on the membrane. The same principle was applied to clostridia (Bisson & Cabelli 1979) and *Aeromonas hydrophila* (Rippey & Cabelli 1979), and can be seen in the confirmation of enterococci by the hydrolysis of aesculin (Anon 1983). This type of test may reduce the total analysis time to 24 h. Biochemical test kits have also been produced with an incubation time of 2 to 4 h. We still, however, rely on presumptive counts for any decisions we may take about water quality.

Utilisation of specific enzymes

The need for less labour intensive analysis, together with utilisation of specific enzymes for easier interpretation and an earlier confirmed result, has lead to a return to the principles of the multiple tube technique. There is a range of chromogenic substrates which can be incorporated into media to give enzyme-based colour changes (Table 1). The enzyme β-galactosidase cleaves lactose into glucose and galactose, and is an essential stage in lactose fermentation. Substrates such as ortho-nitrophenol-β-D-galactopyranoside, when cleaved by the enzyme, produce the yellow colour of ortho-nitrophenol. The enzyme β-galactosidase is found in coliforms, including *E. coli*, and therefore a simple colour test for coliforms becomes available. An additional enzyme, β-glucuronidase, is found in *E. coli*, *Salmonella* spp. and *Shigella* spp. An additional substrate, methyl umbelliferyl-β-D-glucuronide, can also be added to the medium. The substrate is broken down by the enzyme and free methyl umbelliferone can be demonstrated by fluorescence in the medium under ultra-violet light. Edberg *et al.* (1988) developed the combination of the two substrates in a chemically-defined medium, providing the basis for a qualitative detection system for coliforms and *E. coli*. The test, originally a 24 h test and now designed for 18 h incubation, provides a confirmed result, is less labour intensive than membrane filtration, and uses only 100 ml of water. Although originally only qualitative, its use has been validated through extensive trials (Edberg *et al.* 1989; Cowburn *et al.* 1994) and was found to give comparable results to standard methods. The test method has now been modified to provide quantitative results in the form of the Quantitray (Idexx, USA) and national trials are now being run to validate this.

Table 1. *A list of some of the chromogenic and fluorogenic substrates available commercially.*

Para-nitrophenol-β-D-galactopyranoside (PNPG)
Ortho-nitrophenol-β-D-galactopyranoside (ONPG)
4-methylumbelliferyl-β-D-galactopyranoside (MUGAL)
4-methylumbelliferyl-β-D-glucuronide (MUG)
8-hydroxyquinoline-β-glucuronide (BCIG)
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)
4-methylumbelliferyl phosphate (MUP)
5-bromo-4-chloro-3-indolyl phosophate (BCIP)
4-methylumbelliferyl-β-glucoside

There are other chromogenic substrates. Sartory & Howard (1992) described the use of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as a marker for β-glucuronidase in the isolation of *E. coli* from water, in a single membrane filtration test incubated at 37°C. The principle has now been adopted by a number of manufacturers and a wide variety of media are now available containing chromogenic substrates. Some of these are listed in Table 2. Although the test still takes 18 h, analysis is reduced to a single membrane and the results obtained are confirmed results. Like Colilert, the test volume has been reduced to 100 ml.

Table 2. *Some examples of commercially available chromogenic and fluorogenic media.*

Product Name	Type of medium	Manufacturer
Colilert	Liquid	Indexx, USA
Colilert (Quantitray)	Liquid	Indexx, USA
Enterolert	Liquid	Indexx, USA
Colisure	Liquid	Millipore, USA
Coliform	Test kit	Millipore, USA
CHROMagar ECC	Agar	Chromagar, France
CHROMagar Urinary Paths	Agar	Chromagar, France
CHROMagar *Candida*	Agar	Chromagar, France
Fluorocult	Liquid	Merck, Germany
Chromocult Coliform	Agar	Merck, Germany
Chromocult *Enterococcus*	Agar	Merck, Germany
Microsure *E. coli*	Agar	Gelman, USA
Coli I D	Agar	Biomerieux, France
EMX Agar	Agar	Biotest, Germany
CL-EC-MF Agar	Agar	Biolife, Italy

A number of questions have been raised about the ability of environmentally or disinfectant-damaged organisms to grow on selective media with high levels of nutrients, and to express the enzymes β-galactosidase and β-glucuronidase. The addition of sodium pyruvate to isolation media (Sartory 1995) will improve the recovery of organisms. In trials at Yorkshire Environmental as part of a national trial comparing the method of Sartory & Howard (1992) with conventional membrane filtration, a significant number of samples failed on the test medium (m-LGA) with 100 ml of treated water, but were found to be satisfactory by conventional membrane filtration on the same sample (Anon 1983). In addition, there was an increase in the number of coliforms isolated on the new medium compared with the numbers isolated by the standard method when both were positive. Pyruvate was not the only factor in m-LGA that was different but an increased sensitivity was noted.

The expression of the enzyme β-glucuronidase by *E. coli* is variable (Lewis & Mak 1989; Clarke *et al.* 1991; Schets *et al.* 1993). Environmentally-derived *E. coli* from surface waters was found to be poor at expressing β-glucuronidase. This was one point highlighted in a report prepared for the Department of the Environment, on the comparison of a number of media with simulated and environmental samples done by the Public Health Laboratory Service. An additional problem may be environmental coliforms which possess β-galactosidase but fail to ferment lactose on primary isolation. Such isolates would be positive by chromogenic substrate analysis but negative by lactose fermentation. Increasing the sensitivity of the detection method, whilst important in detecting faecal contamination, will apparently lead to a decrease in water quality and in water quality statistics. It is therefore important that new methods are comparable with existing methods in their sensitivity and that this is properly validated. It is equally important to understand that different sources of water may give varying results with chromogenic and fluorogenic media. Trials in any water company are therefore important to assess the sensitivity of any new test method with a cross-section of waters. What then becomes unclear is to what extent the trials should be conducted. How many different water types should be examined and how many samples should be tested for each water type? To compound matters further, potable water analysis produces a low failure rate. Many thousands of samples may need to be analysed to produce a significant number of

positive samples for statistical analysis. The question of the analysis of a single 100 ml water sample instead of 200 ml of water must also be considered carefully, in that any apparent improvement in water quality statistics might be interpreted as being due to a change in the test procedure or a reduction in the test volume.

It is not difficult to produce new media using the widely available chromogenic substrates. For example, detection of *Clostridium perfringens* is based on the production of hydrogen sulphide from the reduction of sulphite, producing black colonies on isolation media. This feature is variable (Oldham 1995; Rushby *personal communication*) and typical colonies are often colourless. Adding BCIP for phosphatase in the selective medium will give blue colonies or MUP will give fluorescent colonies.

Whilst these newer test methods are designed to be less labour intensive (more cost effective) and provide confirmed results, they still require 18 h incubation to produce a result. In some respects this is convenient because samples are analysed on one afternoon and results are available at the beginning of the following working day. Reduction of the analytical time to provide results on the same working day, in the form of a 6 to 8 h test (Sidorowicz & Whitmore 1995), may seem desirable but is not practicable. Without altering current sampling regimes, results would be available about midnight. Reduction of the analysis time to 1 to 2 h would provide same-day results but there is insufficient time for culture. As an alternative, assessment for viability now becomes important. Raw waters contain coliforms and *E. coli*. Treatment is designed to remove some and render the remainder non-viable (non-culturable). These organisms will not be detected by standard cultural techniques but may be detected by viability assessment and their significance needs to be clearly established.

Alternative methods of detection

Newer techniques for detecting microorganisms in drinking water have been reviewed by Sidorowicz & Whitmore (1995). Some of these have limited culture as part of the technique. The short-term culture of target organisms in non-selective culture media (eliminating the need for pre-enrichment) may be followed by a method of labelling cells specifically and a highly sensitive method of detection.

A number of DNA-specific fluorochromes are available for the detection of microorganisms. Some of these can differentiate between viable and non-viable bacteria. Acridine orange is commonly used to detect and count bacteria in water by direct microscopy (Fry 1988). Alternatives are 4'6'-diamidino phenylindole (DAPI) and the bis-benzamide derivative Hoechst 33342, used by Monger & Landry (1993) to detect bacteria in fresh and marine waters. Rhodamine 123 (Kaprelyants & Kell 1993; Morgan *et al.* 1993) and fluorescein diacetate (Jorgensen *et al.* 1992) have been used to determine viable biomass in water and waste water treatment. A tetrazolium salt, 5-cyano-2,3-ditolyltetrazolium chloride (CTC), has been used to detect viable bacteria in culture (Kaprelyants & Kell 1993) and in secondary treated effluents (Rodriguez *et al.* 1992). It has also been used to count planktonic and sessile respiring bacteria in drinking water (Schaule *et al.* 1993). Bovill *et al.* (1994) also reported the use of CTC for detecting metabolic activity in heat-stressed cells. Deere *et al.* (1995) used *bis*-(1,-3-dibutylbarbituric acid) trimethine oxonol for assessing bacterial viability, and Porter *et al.* (1995) describe the use of a range of viability dyes for analysis of indigenous bacteria from soil. The incorporation of additional specific labels in the form of fluorescent antibodies enables specific bacterial species to be detected and their viability assessed.

The fluorochromes listed in Table 3 can be used to study bacteria in water. When results of direct counting are compared with plate counts on nutrient media for the formation of biofilms, two very different pictures emerge. Figure 1 shows the accumulation of bacteria in a biofilm

over a period of 2 weeks. Counts were made using yeast extract agar (3 days at 22°C; Anon 1994), R2A agar (7 days at 20°C; Reasoner & Geldreich 1985) and staining with DAPI. Values obtained by direct counting are always higher than cultural techniques but after 9 days there is a significant increase in the biofilm that can only be detected by DAPI staining.

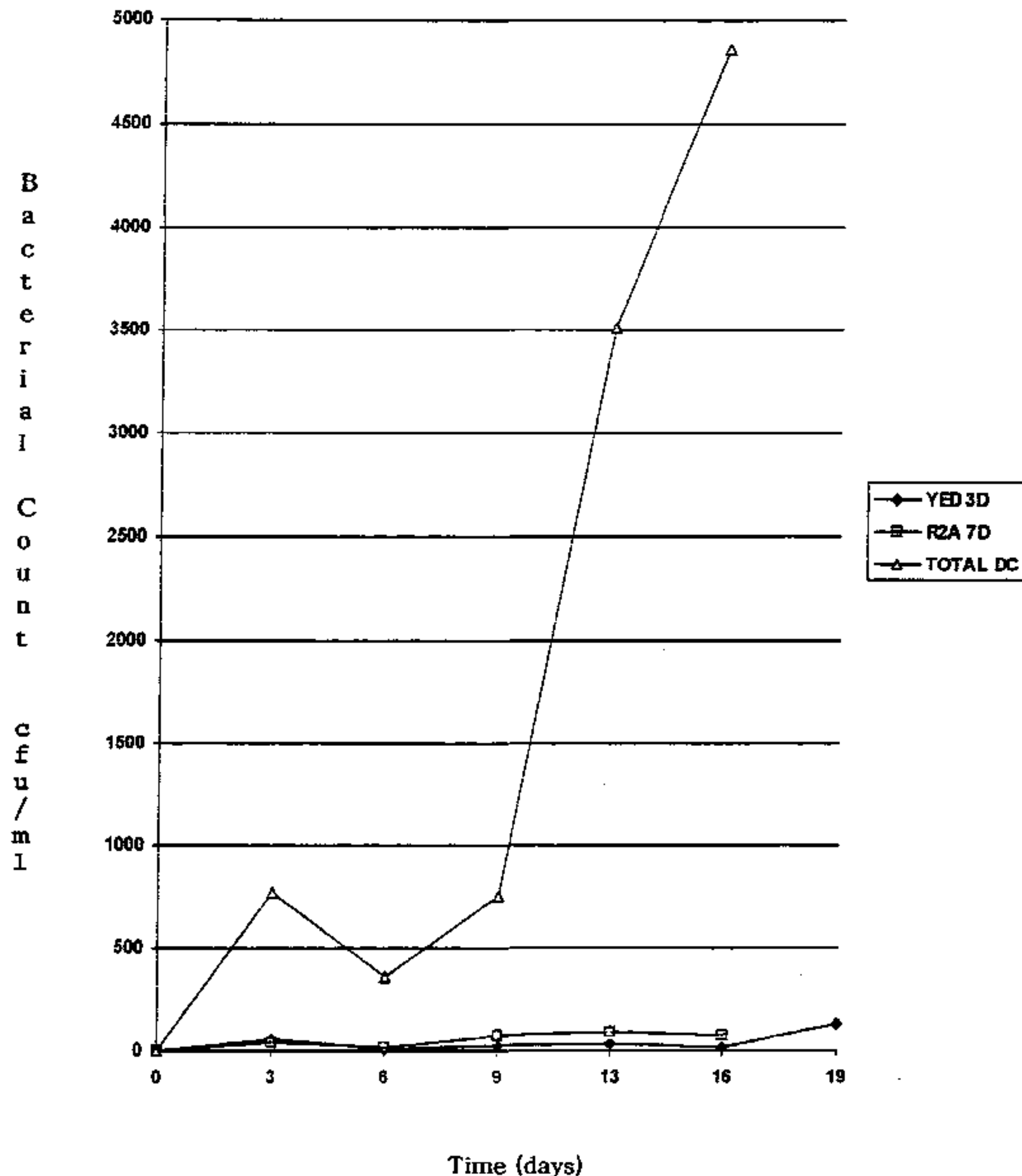

Figure 1. Relationship between bacterial counts (colony-forming units per ml) on nutrient media (yeast extract for 3 days (♦, YED 3D) and R2A agar for 7 days (□, R2A 7D)) and total count using DAPI (△, Total DC).

Table 3. *Some examples of fluorochromes with their fluorescent properties.*

Reagent	Wavelength Excitation	Emission
Acridine orange	487	510
Fluorescein isothiocyanate	488	530
Phycoerythrin (PE)	488	575
Diamidino phenylindole (DAPI)	345	455
Propidium iodide (PI)	493	639
Rhodamine 123	505	543
Fluorescein diacetate (FDA)	505	530
Trimethine oxonol	535	560
Hoechst 33343	350	500
Cyanoditolyl tetrazolium chloride (CTC)	530	600

Immunological methods

Immunofluorescence, based on detection by labelling antibodies with a fluorochrome (direct immunofluorescence) or the detection of an antibody antigen complex with a labelled anti-species (indirect immunofluorescence), has been used widely to detect and serotype specific microorganisms. The development of mouse monoclonal antibodies has further enhanced this technique. Immunofluorescence has been used to detect a wide variety of microorganisms in food and water and provides a quick way of establishing their presence. Colbourne & Dennis (1989) demonstrated *Legionella pneumophila* serogroup 1 in waters abstracted for drinking. Brayton *et al.* (1987) described the enumeration of *Vibrio cholera* in tropical waters using immunofluorescence as a more sensitive tool than culture. More recently Pyle *et al.* (1995) have described a combined immunofluorescence and fluorogenic probe to detect and assess the viability of *E. coli* O157:H7 in water.

Immunofluorescence is one of many methods to be used for the detection of bacteria. Enzyme-linked immunosorbant assay (ELISA) has also been used, after a short period of growth, to detect *Salmonella* spp. in foods. However, with the exception of the detection of rotavirus, the technique is not used in the water industry. The test is relatively insensitive, requiring ca. 10^4 cells for a positive response.

Immunocapture using latex-coated magnetisable beads has shown more promise. Used primarily for the isolation of bacteria from food (Cudjoe *et al.* 1994), the technique has been adapted for the isolation of *E. coli* O157:H7 from food (Wright *et al.* 1994) and water, and has been used to concentrate *Cryptosporidium* from water. The principle is based on using antibody-coated magnetisable particles to capture target organisms from a heterogenous mixture of microorganisms. Once the organisms are captured, the beads can be separated from the mixture using a magnet. The captured organisms can be subjected to conventional culture or detected by microscopy, ELISA, molecular techniques or ATP assay. The period of culture by pre-enrichment before detection can be substantially reduced to ca. 4 to 5 h incubation, where only low numbers of organisms are present. With rapid detection techniques, organisms can be detected in as little as 8 hours.

Flow cytometry

Flow cytometry was originally developed for use in the study of eukaryotic cells and, in particular, leucocytes in mammalian blood. More recently attention has been turned to the study of microorganisms. Flow cytometry has been used in the study of the bacterial cell cycle (Skarstad *et al.* 1983) and its potential as a tool for microbial ecology has been recognised by

Edwards *et al.* (1992) who review the principles of flow cytometry. Diaper & Edwards (1994) reported the use of flow cytometry for the detection and enumeration of viable *Staphylococcus aureus* during survival in a lakewater microcosm, and Porter *et al.* (1995) were able to enumerate and sort mixtures of *S. aureus* and *E. coli* labelled with fluorescent antibody.

Flow cytometry can be used as a rapid and sensitive method for the analysis of bacterial populations and for detecting small numbers of target microorganisms within a heterogenous population (Watkins *et al.* 1995). There is a wide range of fluorescent probes available (Table 3) for both total and viable counts, and for specific detection using labelled monoclonal or polyclonal antibodies. In comparing flow cytometric counting with direct epifluorescence counting on the same suspension, results would appear to be reproducible under the correct operating conditions (Table 4). Detection of small numbers of target organisms was demonstrated by Pinder *et al.* (1994), counting *Aeromonas salmonicida* in survival studies and detecting *Salmonella typhimurium* in a mixed *Salmonella* population. Industrial applications include analysis of yeast in wine and fruit preparations, total viable counts on vegetables, and antibiotic sensitivity testing (Brailsford & Gatley 1994). Studies on drinking water, by comparing propidiun iodode (PI) staining and counting by epifluorescence, and PI staining and counting by flow cytometry, have given good comparative results (Table 5).

Table 4. *Comparison of counts of* Staphylococcus aureus *stained with CTC and DAPI, obtained by flow cytometry (FCM) and by sorting and direct counting (EFM).*

CTC Stain		DAPI Stain	
FCM	EFM	FCM	EFM
68	68	8	6
46	44	7	6
41	39	12	12
65	62	15	10
256	257	31	33

Table 5. *Comparison of counts on drinking water samples stained with propidium iodide and enumerated by direct epifluorescence microscopy (EFM) and flow cytometry (FCM).*

Sample Number	Total Count (cells per ml)	
	FCM	EFM
16113433	880,000	760,000
16113343	70,000	28,000
16113435	168,000	117,000
16113436	560,000	504,000
16113444	284,000	212,000

There is a wide range of applications for rapid-flow cytometric analysis following a short period of pre-enrichment. *Salmonella* and *Listeria monocytogenes* could be analysed simultaneously from the same broth using antibodies conjugated with different fluorochromes. The incubation period could be as little as 4 h, and with a sort facility target organisms can be sorted for culture, ELISA, or confirmation with molecular probes. Rapid detection of *Legionella pneumophila* from environmental samples also becomes easy and determination of viability is easy. Having a short period of culture followed by flow cytometric analysis and confirmation, immunological or molecular techniques will reduce analysis time down to less than 8 h.

Discussion

A number of rapid methods are available for the detection and enumeration of microorganisms. Most of these have been developed for, and are used in, the food industry. In the water industry, current attention is focused on chromogenic and fluorogenic substrates as a way of getting confirmed results after primary incubation. A similar trend is developing in clinical microbiology. To be effective in water analysis, a reduction to 8 h for the time between sampling and result is not really applicable. A reduction to between 1 and 2 h is a possibility but culture becomes impossible and we have to rely on enzyme-based detection methods. There are substrates for the enzymes β-galactosidase and β-glucuronidase which emit light when broken down. Detection systems for light emission can detect single cells on a membrane and can therefore detect faecal indicators without growth.

Direct comparisons with cultural techniques are important if meaningful comparisons are to be made about water quality. The technology is available and being developed, and perhaps one day microbiologists will have to accept a change in culture, throwing away the humble Petri dish for rapid detection of microorganisms "*in-situ*".

The authors wish to thank Alcontrol UK for permission to publish this article. The opinions expressed here are those of the authors and not necessarily those of the company they represent. For Harry Fennell and Frank Jones.

References

Anon, (1956). *The Bacteriological Examination of Water Supplies.* Report No. **71**. Ministry of Health, HMSO, London.

Anon, (1983). *The Bacteriological Analysis of Drinking Water Supplies 1982.* Reports on Public Health & Medical Subjects No. **71**. Department of the Environment, HMSO, London.

Anon, (1994). *The Microbiology of Water 1994. Part 1 – Drinking Water.* Reports on Public Health & Medical Subjects No. **71**. HMSO, London.

Bisson, J. W. & Cabelli, V. J. (1979). Membrane filter enumeration method for *Clostridium perfringens. Applied & Environmental Microbiology*, **37**, 55-66.

Bovill, R. A., Shallcross, J. A. & Mackey, B. M. (1994). Comparison of the fluorescent redox dye 5-cyano-2,3-ditolyltetrazolium chloride with p-iodonitrotetrazolium violet to detect metabolic activity in heat-stressed *Listeria monocytogenes* cells. *Journal of Applied Bacteriology*, **77**, 353-358.

Brailsford, M. A. & Gatley, S. (1994). Industrial application of flow cytometry for the rapid detection of microorganisms. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Brayton, P., Tamplin, M., Huq, A. & Colwell, R. R. (1987). Enumeration of *Vibrio cholera* O1 in Bangladesh waters by fluorescent-antibody direct viable count. *Applied & Environmental Microbiology*, **53**, 2862-2865.

Clarke, D. L., Milner, B. B., Stewart, M. H., Wolfe, R. L. & Olson, B. H. (1991). Comparative study of commercial 4-methylumbelliferyl-β-D-glucuronide preparations with the standard methods membrane filtration faecal coliform test for the detection of *Escherichia coli* in water samples. *Applied & Environmental Microbiology*, **57**, 1528-1534.

Colbourne, J. S. & Dennis, P. J. (1989). The ecology and survival of *Legionella pneumophila. Journal of the Institution of Water Engineers & Scientists*, **3**, 345-350.

Cowburn, J. K., Goodall, T., Fricker, E. J., Walter, K. S. & Fricker, C. R. (1994). A preliminary study of the use of Colilert for water quality monitoring. *Letters in Applied Microbiology*, **19**, 52-54.

Cudjoe, K. S., Patel, P. D., Olsen, E., Skjerve, E. & Olsvik, O. (1994). Immunomagnetic separation techniques for the detection of pathogenic bacteria in foods. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Deere, D., Porter, J., Edwards, C. & Pickup, R. W. (1995). Evaluation of the suitability of *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol, (biBA-C_4(3)-), for the flow cytometric assessment of bacterial viability. *FEMS Microbiology Letters*, **130**, 165-170.

Diaper, J. P. & Edwards, C. (1994). Survival of *Staphylococcus aureus* in lakewater monitored by flow cytometry. *Microbiology*, **140**, 35-42.

Dufour, A. P. & Cabelli, V. J. (1975). Membrane filter procedure for enumerating the component genera of the coliform group in sea water. *Applied & Environmental Microbiology*, **29**, 826-833.

Edberg, S. C., Allen, M. J. & the National Collaborative Study. (1988). National field evaluation of a defined substrate

method for the simultaneous enumeration of coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. *Applied & Environmental Microbiology,* **54**, 1595-1601.

Edberg, S. C., Allen, M. J., Smith, D. B. & the National Collaborative Study. (1989). National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with presence-absence techniques. *Applied & Environmental Microbiology,* **55**, 1003-1008.

Edwards, C., Porter, J., Saunders, J. R., Diaper, J. *et al.* (1992). Flow cytometry and microbiology. *Society for General Microbiology Quarterly,* **November 1992**, 105-108.

Fry, J. C. (1988). Determination of biomass. In *Methods in Aquatic Bacteriology* (ed. B. J. Austin), pp. 27-72. Wiley, New York.

Jorgensen, P. E., Eriksen, T. & Jensen, B. K. (1992). Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilisation rate and FDA hydrolysis. *Water Research,* **26**, 1495-1501.

Kaprelyants, A. S. & Kell, D. B. (1993). Dormancy in stationary-phase cultures of *Micrococcus luteus;* flow cytometry analysis of starvation and resuscitation. *Applied & Environmental Microbiology,* **59**, 10, 3187-3196.

Lewis, C. M. & Mak, J. L. (1989). Comparison of membrane filtration and autoanalysis Colilert presence absence techniques for the analysis of total coliforms and *Escherichia coli* in drinking water samples. *Applied & Environmental Microbiology,* **55**, 3091-3094.

Monger, B. C. & Landry, M. R. (1993). Flow cytometric analysis of marine bacteria with Hoechst 33342. *Applied & Environmental Microbiology,* **59**, 905-919.

Morgan, J. A. W., Rhodes, G. & Pickup, R. W. (1993). Survival of nonculturable *Aeromonas salmonicida* in lake water. *Applied & Environmental Microbiology,* **59**, 874-880.

Oldham, N. S. (1995). *A comparison of methods for the detection of* Clostridium perfringens *in raw water samples.* Internal project report, LabServices, Bradford.

Pinder, A. C., Edwards, C., Clarke, R. G., Diaper, J. P. & Poulter, S. A. G. (1994). Detection and enumeration of viable bacteria by flow cytometry. In *New Techniques in Food and Beverage Microbiology* (eds R. G. Kroll, A. Gilmour & M. Sussman), pp. 131-145. Blackwell Scientific Publications, Oxford.

Porter, J., Pickup, R. & Edwards, C. (1995). Flow cytometric methods for the detection and viability assessment of bacteria in soil. (In press).

Public Health Laboratory Service. (1969). A minerals-modified glutamate medium for the enumeration of coliform organisms in water, by the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies. *Journal of Hygiene,* **81**, 367-374.

Pyle, B. H., Broadway, S. C. & McFeters, G. A. (1995). A rapid, direct method for enumerating respiring enterohaemorrhagic *Escherichia coli* O157:H7 in water. *Applied & Environmental Microbiology,* **61**, 2614-2619.

Reasoner, D. J. & Geldreich, E. E. (1985). A new medium for the enumeration and sub-culture of bacteria from potable water. *Applied & Environmental Microbiology,* **49**, 1-7.

Rippey, S. R. & Cabelli, V. J. (1979). Membrane filter procedure for the enumeration of *Aeromonas hydrophila* in fresh water. *Applied & Environmental Microbiology,* **38**, 108-113.

Rodriguez, G. G., Phipps, D., Ishiguro, K. & Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualisation of actively respiring bacteria. *Applied & Environmental Microbiology,* **58**, 1801-1808.

Sartory, D. P. (1995). Improved recovery of chlorine-stressed organisms with pyruvate supplemented media. *Water Science & Technology,* **31**, 255-258.

Sartory, D. P. & Howard, L. (1992). A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms in drinking water. *Letters in Applied Microbiology,* **15**, 273-276.

Schaule, G., Flemming, H. C. & Ridgway, H. F. (1993). Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Applied & Environmental Microbiology,* **59**, 11, 3850-3857.

Schets, F. M., Medema, G. J. & Havelaar, A. H. (1993). Comparison of Colilert with Dutch standard methods for *Escherichia coli* and total coliforms in water. *Letters in Applied Microbiology,* **17**, 17-19.

Sidorowicz, S. V. & Whitmore, T. N. (1995). Prospects for new techniques for rapid bacteriological monitoring of drinking water. *Journal of the Institute of Water & Environment Management,* **9**, 92-98.

Skarstad, K., *et al.* (1983). Cell cycle parameters of slowly growing *E. coli* studied by flow cytometry. *Journal of Bacteriology,* **154**, 656-662.

Watkins, J., Kemp, P. & Shepherd, K. (1995). Analysis of water samples for *Cryptosporidium* including the use of flow cytometry. In *Protozoan Parasites and Water* (eds W. B. Betts *et al.*), pp. 115-121. Royal Society of Chemistry.

Windle Taylor, E., Burman, N. P. & Oliver, C. W. (1953). Use of the membrane filter in the bacteriological examination of water. *Journal of Applied Chemistry,* **3**, 233.

Wright, D. J., Chapman, P. A. & Siddons, C. A. (1994). Immunomagnetic separation as a method for isolation *Escherichia coli* O157:H7 from food samples. *Epidemiology & Infection,* **113**, 31-39.