



ENVIRONMENT AGENCY

**The Microbiology of Drinking Water (2004) – Part 6 – Methods for the
isolation and enumeration of sulphite-reducing clostridia
and *Clostridium perfringens* by membrane filtration**

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2004) - Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration

Methods for the Examination of Waters and Associated Materials

This booklet contains two methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration, and supercedes “The Microbiology of Drinking Water (2002) - Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration”.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other booklets include

- The Microbiology of Drinking Water (2002) -
- Part 1 - Water quality and public health
- Part 2 - Practices and procedures for sampling
- Part 3 - Practices and procedures for laboratories
- Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
- Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration
- Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
- Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas* by membrane filtration
- Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and most probable number techniques
- Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment and membrane filtration

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials"

and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

- 1 General principles of sampling and accuracy of results
- 2 Microbiological methods
- 3 Empirical and physical methods
- 4 Metals and metalloids
- 5 General non-metallic substances
- 6 Organic impurities
- 7 Biological methods
- 8 Biodegradability and inhibition methods
- 9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods and details on how to obtain copies are available from the Agency's internet web-page (www.environment-agency.gov.uk/nls) or from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

October 2003

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1999 (SI 1999/437). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

A Enumeration of sulphite-reducing clostridia by membrane filtration

A1 Introduction

Tests for sulphite-reducing clostridia play only a subsidiary role in water examination. The organisms form spores which are environmentally resistant and their presence may indicate soil contamination, although some species may grow in deposits, and be associated with corrosion of distribution pipes. *Clostridium perfringens* is a sulphite-reducing species and is associated with faecal contamination. The significance of sulphite-reducing clostridia and *Clostridium perfringens* in water treatment and supply are described elsewhere⁽¹⁾ in this series.

A2 Scope

The method is suitable for the examination of drinking waters including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions⁽²⁾.

A3 Definitions

Sulphite-reducing clostridia are Gram-positive anaerobic spore-forming rods, which in the context of this method reduce sulphite to sulphide at 37 °C within 24 hours.

A4 Principle

A volume of sample is filtered and the membrane filter placed on the surface of an agar medium containing sulphite and iron(III). The agar medium is then incubated under anaerobic conditions at 37 °C. Sulphite-reducing clostridia usually produce black colonies as a result of the reduction of sulphite to sulphide which reacts with the iron(III) salt. If only a spore count is required then the sample is heat treated at 60 °C prior to filtration in order to kill vegetative bacteria.

A5 Limitations

The method is suitable for most types of aqueous samples except those with high turbidities which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

A6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations⁽³⁾ and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere⁽²⁾ in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere ⁽²⁾ in this series. Principally appropriate membrane filtration apparatus and incubators (fan assisted, static temperature) are required. Other items include:

- A7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent).
- A7.2 Incubator capable of maintaining a temperature of 37 ± 1.0 °C.
- A7.3 Anaerobic jars, or similar equipment, and anaerobic gas-generating system (for generating atmospheres of approximately 9 - 13 % carbon dioxide).
- A7.4 Filtration apparatus, sterile filter funnels, and source of vacuum.
- A7.5 Sterile membrane filters, for example, white, 47 mm diameter cellulose-based, 0.45 µm nominal pore size.
- A7.6 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations in their formulation. The performance of all media and reagents should be verified prior to use in this method. Variations in the preparation and storage of media should also be verified.

Unless otherwise stated chemical constituents are added as anhydrous salts.

A8.1 *Tryptose sulphite cycloserine agar without egg yolk*^(4, 5)

Yeast extract	5 g
Tryptose	15 g
Soya peptone	5 g
Sodium metabisulphite	1 g
Iron(III) ammonium citrate	1 g
Agar	14 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Allow the medium to cool to 45 - 48 °C. Add 4 ml of a filter-sterilised solution of D-cycloserine in distilled water at a concentration of 100 mg ml⁻¹. Mix thoroughly, and dispense into Petri dishes. The final pH of the medium should be 7.6 ± 0.2.

Performance of the medium deteriorates during storage due to exposure to oxygen. Prepared media may be stored in a refrigerator under anaerobic conditions at a temperature between 2 - 8 °C for up to one week. However, when fresh medium is used, the colony

characteristics that are observed tend to be more defined. The medium, once removed from the refrigerator, should be discarded if not used.

A8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Ringer's solution and maximum recovery diluent.

A9 **Analytical procedure**

A9.1 *Sample preparation*

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on one of the membrane filters is likely to fall within this range. For treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

If it is the intention to count only the spores of sulphite-reducing clostridia then the volume of sample should be heated to 60 ± 2 °C (for example, in a water bath) and maintained at this temperature for 15 ± 1 minutes. The temperature may be monitored by placing an appropriate thermometer in a bottle containing the same volume of water as the sample volume being treated.

A9.2 *Sample processing*

Place the sterile filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a well-dried tryptose sulphite cycloserine agar Petri dish. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped.

As the spores of sulphite-reducing clostridia are very resilient, funnels that have been used once should be sterilised by autoclaving before being used again. Placing funnels in a water bath at this stage may not be sufficient to kill spores. If different volumes of the same sample are to be examined, the funnel may be re-used without sterilising the funnel provided that the smallest volume, or highest dilution of the sample, is filtered first. For different samples, take a fresh pre-sterilised funnel and repeat the filtration process. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes

contaminated or a membrane filter becomes damaged. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

Incubate the Petri dishes at 37 °C in an anaerobic jar or similar system containing an indicator of anaerobiosis and an atmosphere containing 9 - 13 % carbon dioxide. Examine the dishes after 21 ± 3 hours incubation.

A9.3 *Reading of results*

After incubation, count all black colonies.

A9.4 *Confirmation tests*

The specificity of tryptose sulphite cycloserine agar is such that confirmation of isolates is not usually required.

A10 **Calculations**

A10.1 Confirmed sulphite-reducing clostridia

The number of confirmed sulphite-reducing clostridia colonies is generally quoted as the number of colonies per 100 ml. Calculate the confirmed count as follows:

$$\text{Confirmed count/100 ml} = \frac{\text{Number of colonies} \times 100}{\text{Volume of sample filtered (ml)}}$$

A11 **Expression of results**

Counts for sulphite-reducing clostridia are expressed in colony forming units per volume of sample. For drinking water, the volume is typically 100 ml.

A12 **Quality assurance**

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Clostridium perfringens*) and non-target bacteria (for example *Bacillus* species). Petri dishes should be incubated for 21 ± 3 hours at 37 °C. Further details are given elsewhere⁽²⁾ in this series.

A13 **References**

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437, The Stationery Office.
4. Enumeration of food-borne *Clostridium perfringens* in egg yolk free tryptose-sulphite-cycloserine agar, *Applied Microbiology*, A H W Hauschild and R Hillsheimer, 1974, **27**, pp521-526.
5. Membrane filtration enumeration of faecal clostridia and *Clostridium perfringens* in water, *Water Research*, D P Sartory, 1986, **20**, pp1255-1260.

B Enumeration of *Clostridium perfringens* by membrane filtration

B1 Introduction

Tests for *Clostridium perfringens* play only a subsidiary role in water examination. The organisms form spores which are resistant to environmental stress and can persist in the environment for some time. *Clostridium perfringens* is associated with faecal contamination. If found at a time when other faecal indicator organisms are no longer detectable, the organism may indicate remote or intermittent pollution. The monitoring of *Clostridium perfringens* during water treatment processes may be useful in assessing the performance of such treatment. The significance of *Clostridium perfringens* in water treatment and supply are described elsewhere⁽¹⁾ in this series.

B2 Scope

The method is suitable for the examination of drinking waters including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

Users wishing to employ this method should verify its performance under their own laboratory conditions⁽²⁾.

B3 Definitions

Clostridium perfringens is a Gram-positive anaerobic spore-forming rod, which in the context of this method reduces sulphite to sulphide at 44 °C within 24 hours. *Clostridium perfringens* is non-motile, reduces nitrate, ferments lactose and liquefies gelatin.

B4 Principle

A volume of sample is filtered and the membrane filter placed on the surface of an agar medium containing sulphite and iron(III). The agar medium is incubated under anaerobic conditions at 44 °C. *Clostridium perfringens* usually produces black colonies as a result of the reduction of sulphite to sulphide which reacts with the iron(III) salt. If only a spore count is required, then the sample is heat treated at 60 °C prior to filtration in order to kill vegetative bacteria.

B5 Limitations

The method is suitable for most types of aqueous samples except those with high turbidities which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The maximum number of colonies that should be counted from a single membrane is approximately 100.

B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations⁽³⁾ and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere⁽²⁾ in this series.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere⁽²⁾ in this series. Principally appropriate membrane filtration apparatus and incubators (fan assisted, static temperature) are required. Others items include:

- B7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent).
- B7.2 Incubators capable of maintaining temperatures of 37 ± 1.0 °C and 44 ± 0.5 °C.
- B7.3 Anaerobic jars, or similar equipment, and anaerobic gas-generating system (for generating atmospheres of approximately 9 - 13 % carbon dioxide).
- B7.4 Filtration apparatus, sterile filter funnels, and source of vacuum.
- B7.5 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size.
- B7.6 Smooth-tipped forceps.

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations in their formulation. The performance of all media and reagents should be verified prior to use in this method. Variations in preparation and storage of media should also be verified.

Unless otherwise stated chemical constituents are added as anhydrous salts.

B8.1 *Tryptose sulphite cycloserine agar without egg yolk* ^(4,5)

Yeast extract	5 g
Tryptose	15 g
Soya peptone	5 g
Sodium metabisulphite	1 g
Iron(III) ammonium citrate	1 g
Agar	14 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients in the water and dissolve by heating and stirring. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Allow the medium to cool to 45 - 48 °C. Add 4 ml of a filter-sterilised solution of D-cycloserine in distilled water at a concentration of 100 mg ml⁻¹. Mix thoroughly, and dispense into Petri dishes. The final pH of the medium should be 7.6 ± 0.2.

Performance of the medium deteriorates during storage due to exposure to oxygen. Prepared media may be stored in a refrigerator under anaerobic conditions at a temperature between 2 - 8 °C for up to one week. However, when fresh medium is used, the colony

characteristics that are observed tend to be more defined. Medium, once removed from the refrigerator, should be discarded if not used.

B8.2 *Buffered nitrate-motility medium*⁽⁶⁾

Beef extract	3 g
Peptone	5 g
Potassium nitrate	5 g
D-Galactose	5 g
Glycerol	5 g
Disodium hydrogen phosphate	2.5 g
Agar	3 g
Distilled, deionised or similar grade water	1 litre

Dissolve the solid ingredients in 950 ml of water by heating to boiling point whilst stirring continuously. Dissolve the glycerol in 50 ml of water in a separate container and add this solution to the base medium and mix thoroughly. Dispense the resulting solution in 10 ml aliquots in appropriately sized capped tubes. Sterilise the medium by autoclaving at 121 °C for 15 minutes. The final pH of the medium should be 7.3 ± 0.2 . Prepared tubes may be stored at a temperature between 2 - 8 °C for up to one month if protected against dehydration.

Before use, stored media should be heated for 10 - 15 minutes in a boiling water bath, to ensure that the contents have melted and to eliminate any absorbed oxygen. The tubes are then allowed to cool and the media to solidify ready for use.

B8.3 *Nitrate reduction test reagents*⁽⁷⁾

Reagent A	
Sulphanilic acid	0.8 g
5N acetic acid	100 ml

Warm gently to aid dissolving.

Reagent B	
N, N-dimethyl 1-naphthylamine	0.6 ml
5N acetic acid	100 ml

Dissolve the amine in the acetic acid solution. Warm gently to aid dissolving.

The reagents are stable for several months when stored at a temperature between 2 - 8 °C.

For the combined reagent, mix equal volumes of reagents A and B immediately prior to use. Prepare in small volumes sufficient for the tests to be performed. The combined reagent may be stored at a temperature between 2 - 8 °C, protected from direct light, and used within 24 hours.

B8.4 *Lactose-gelatin medium*⁽⁴⁾

Tryptose	15 g
Yeast extract	10 g
Disodium hydrogen phosphate	5 g
Gelatin	120 g
Lactose	10 g
Phenol red (0.4 % m/v solution)	12.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the gelatin, lactose and phenol red, in the water. Add the gelatin gradually whilst stirring continuously and warming gently to aid dissolving. Adjust the pH to 7.5 ± 0.2 . Add the lactose and phenol red and mix thoroughly to dissolve. Dispense the resulting solution in 10 ml aliquots in appropriately sized capped tubes and sterilise the medium at 121 °C for 15 minutes. The final pH should be 7.5 ± 0.2 . Prepared media may be stored at a temperature between 2 - 8 °C for up to one month if protected against dehydration.

Before use stored media should be heated for 10 - 15 minutes in a boiling water bath, to ensure that the contents have melted and to eliminate any absorbed oxygen. The tubes are then allowed to cool and the media to solidify ready for use.

B8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include zinc powder, Ringer's solution and maximum recovery diluent.

B9 Analytical procedure

B9.1 *Sample preparation*

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on one of the membrane filters is likely to fall within this range. For treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

If it is the intention to count only the spores of *Clostridium perfringens* then the volume of sample should be heated to 60 ± 2 °C (for example in a water bath) and maintained at this temperature for 15 ± 1 minutes. The temperature may be monitored by placing an appropriate thermometer in a reference bottle containing the same volume of water as the sample being treated.

B9.2 *Sample processing*

Place the sterile filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, on the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength

Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids dispersion of the bacteria over the entire surface of the membrane filter during filtration. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a well-dried tryptose sulphite cycloserine agar Petri dish. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped.

As the spores of *Clostridium perfringens* are very resilient, funnels that have been used once should be sterilised by autoclaving before being used again. Placing funnels in a water bath at this stage may not be sufficient to kill spores. If different volumes of the same sample are to be examined, the funnel may be re-used without sterilising the funnel provided that the smallest volume, or highest dilution of sample, is filtered first. For different samples, take a fresh pre-sterilised funnel and repeat the filtration process. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

Incubate the Petri dishes at 44 °C in an anaerobic jar or similar system containing an indicator of anaerobiosis and an atmosphere containing 9 - 13 % carbon dioxide. Examine the dishes after 21 ± 3 hours incubation.

B9.3 *Reading of results*

Under good anaerobic conditions at 44 °C colonies of clostridia are typically black or grey in colour. However, on occasion colourless colonies may be encountered. All colonies growing on tryptose sulphite cycloserine agar at 44 °C should, therefore, be counted as presumptive *Clostridium perfringens*.

B9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of colonies. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be sub-cultured if more than ten are present.

For each isolate, inoculate a tube of buffered nitrate-motility medium by stabbing the medium with a straight wire to just above the bottom of the tube and incubate anaerobically at 37 °C for 21 ± 3 hours. To test for nitrate reduction, add a few drops, approximately 0.2 - 0.5 ml, of the combined nitrate reduction test reagent to each tube. A red colour forming within 15 minutes indicates nitrate reduction to nitrite and the test is regarded as being positive. If a red colour does not develop within this time, add a small amount of zinc powder and leave to stand for 10 minutes. If after this time there is still no red colour this indicates that nitrate has been reduced to nitrite, which has been further reduced. The test is regarded as being positive. However, if a red colour subsequently develops after the addition

of zinc powder, this indicates that nitrate has not been reduced and the test is regarded as being negative.

In addition, inoculate a tube of lactose-gelatin medium by stabbing the medium with a straight wire and incubate anaerobically at 37 °C for 44 ± 4 hours. After incubation the medium will be liquid, irrespective of whether gelatin liquefaction has occurred or not. In order to establish whether gelatin liquefaction has occurred the tubes should be placed in a refrigerator for at least one hour. Gelatin liquefaction will have occurred in tubes where the medium remains liquid after refrigeration.

If necessary, the tubes may be examined after incubating at 37 °C for 21 ± 3 hours and refrigerated (for example, for about one hour) and if gelatin liquefaction occurs, i.e. the test is regarded as positive, the result is recorded. If negative, i.e. the medium remains solid after refrigeration, the tubes should be returned to the incubator. Incubation should be continued until the total incubation period of 44 ± 4 hours has been achieved. The tubes are then re-examined.

A set of control tubes inoculated with appropriate positive and negative strains should be incubated and tested in parallel.

Clostridium perfringens is confirmed by the following reactions:

- (i) Non-motile - growth along the line of the stab and not spread through buffered nitrate-motility medium.
- (ii) Nitrate reduction - red colour after addition of combined nitrate reduction test reagent to buffered nitrate-motility medium, or remaining colourless after addition of zinc powder.
- (iii) Lactose fermentation - orange/yellow colouration of lactose-gelatin medium.
- (iv) Gelatin liquefaction - contents of the lactose-gelatin medium tube become liquefied.

Further identification may be carried out by means of appropriate biochemical and other tests. Suitable commercial identification kits may be used following appropriate performance verification at the laboratory.

B10 Calculations

B10.1 Presumptive *Clostridium perfringens*

The number of presumptive *Clostridium perfringens* colonies is generally quoted as the number of colonies per 100 ml. Calculate the presumptive count as follows:

$$\text{Presumptive count/100 ml} = \frac{\text{Number of colonies} \times 100}{\text{Volume of sample filtered (ml)}}$$

B10.2 Confirmed *Clostridium perfringens*

The number of confirmed *Clostridium perfringens* colonies is calculated by multiplying the number of presumptive *Clostridium perfringens* by the proportion of the isolates that are non-motile, reduce nitrate, ferment lactose and liquefy gelatin.

B11 Expression of results

The number of presumptive and confirmed *Clostridium perfringens* is expressed in colony forming units per volume of sample. For drinking waters, the volume is typically 100 ml.

B12 Quality assurance

New batches of isolation medium should be tested with appropriate reference strains of target bacteria (for example *Clostridium perfringens*) and non-target bacteria (for example *Bacillus* species). New batches of confirmatory media and reagents should be tested with appropriate reference strains of bacteria chosen to verify a positive and a negative reaction in each case. Petri dishes should be incubated for 21 ± 3 hours at 37 °C or 44 °C as appropriate. Further details are given elsewhere⁽²⁾ in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437, The Stationery Office.
4. Enumeration of food-borne *Clostridium perfringens* in egg yolk free tryptose-sulphite-cycloserine agar, *Applied Microbiology*, A H W Hauschild and R Hillsheimer, 1974, **27**, pp521-526.
5. Membrane filtration enumeration of faecal clostridia and *Clostridium perfringens* in water, *Water Research*, D P Sartory, 1986, **20**, pp1255-1260.
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However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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the 1990s, the number of people in the world who are illiterate has increased from 400 million to 600 million.

There are many reasons for this. One is that the population of the world is growing so fast that the number of people who are illiterate is increasing. Another reason is that the quality of education is so poor that many people who are literate are unable to read and write.

There are many ways to improve the quality of education. One way is to invest more money in education. Another way is to train teachers better. A third way is to make sure that all children have access to schools.

It is important to improve the quality of education because it is the only way to reduce poverty and improve the lives of people in the world.

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