Environment Agency

The Microbiology of Drinking Water (2007)- Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains a method for the isolation and enumeration of aerobic spore-forming bacteria by membrane filtration.

Within this series there are separate booklets, each 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 aeruginosa* by membrane filtration
Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

The Microbiology of Drinking Water (2004) -
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for the isolation and enumeration of micro-organisms associated with taste, odour and related aesthetic problems

The Microbiology of Drinking Water (2006) –
Part 5 - The isolation and enumeration of *enterococci* by membrane filtration
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

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. Details of this method are included for information purposes only. Information on the routine use of this method would be welcomed to assess its full capabilities.

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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, soils (including contaminated land) 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. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (www.environment-agency.gov.uk/nls) or by post.

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
July 2006

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 2002 (SI 2002/2677). 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.

The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

Details of this method are included for information purposes only. Information on the routine use of this method would be welcomed to assess its full capabilities. It has not been subjected to widespread use or verification of its performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. This is especially so in relation to its use as a surrogate test for the removal of pathogenic organisms following treatment.

1 Introduction

This method enables a count to be obtained of readily culturable aerobic spore-forming bacteria present in water. These bacteria, primarily species of *Bacillus*, are potentially useful as surrogate indicators for monitoring the performance of water treatment works. They have been used for the assessment of the risk of breakthrough of some pathogenic protozoa, for example oocysts of *Cryptosporidium*[1]. Aerobic bacterial spores occur in large numbers in surface waters, and may be removed to a similar extent as pathogenic protozoa during drinking water treatment. This method offers a quick and simple alternative to more comprehensive tests for pathogenic organisms.

The absence (or significant reduction in numbers) of these bacteria in the final water of surface water treatment works compared to the number of these bacteria in the raw water may, therefore, indicate that the treatment process is operating satisfactorily. Since this test is only a surrogate test for pathogenic organisms, the absence of aerobic spore-forming bacteria does not indicate the absence of pathogenic organisms.

2 Scope

The method is particularly suitable for the examination of final waters from treatment works, but can include samples from all stages of treatment and distribution, those source waters of moderate turbidity, and those waters possessing high turbidities that can be reduced by dilution.

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

3 Definitions

In the context of this method, aerobic spore-forming bacteria are those bacteria that, after heat treatment, form colonies on non-selective nutrient enriched agar medium containing bromothymol blue.

4 Principle

A known volume of sample is heated at 60 °C for 20 minutes to kill any vegetative cells. Following heat treatment, the sample, or dilution of sample is filtered through a membrane filter, which is subsequently placed on the surface of a non-selective nutrient enriched agar medium containing bromothymol blue. Typically, the majority of colonies that develop take up the bromothymol blue giving them a green colour and making them easier to count. However, unstained and other coloured colonies may also be present. Membrane filters

are incubated at 37 °C for 24 hours after which all colonies, irrespective of colour, are counted.

5 Limitations

This 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 target organisms. The maximum number of colonies that should be counted from a single membrane filter is approximately 100. Counts can be obtained from membrane filters containing more than 100 colonies providing that isolated colonies are present and that a hand lens, or similar magnifying aid, is used. Counts obtained in this way should be reported as an estimated count.

6 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.

7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2]. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

7.1 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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

7.2 Incubator capable of maintaining a temperature of 37 ± 1 °C.

7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.45 μm nominal pore size. Membrane filters printed with a grid, as an aid to counting, have been shown to be particularly useful.

7.5 Smooth-tipped forceps.

7.6 Water bath capable of maintaining a temperature of 60 ± 2 °C.

8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in the method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted

accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

8.1 *Nutrient agar supplemented with bromothymol blue*[4]

Yeast extract	2.0 g
Peptone	5.0 g
'Lab-Lemco' powder	1.0 g
Sodium chloride	5.0 g
Bromothymol blue (2 % m/v solution)	2.5 ml
Agar	12.0 g

A stock solution of bromothymol blue should be prepared by dissolving 0.2 g of bromothymol blue in 9 ml of water and 1 ml of 1 M sodium hydroxide solution.

Suspend the ingredients in 1 litre of water and heat to dissolve all the constituents. Dispense the solution into appropriate volumes and sterilise by autoclaving at 121 °C for 15 minutes. Allow the solution to cool. The final pH value of the solution should be 7.4 ± 0.2. Dispense the solution into Petri dishes, for example 50 - 55 mm in diameter. Prepared dishes may be stored at temperatures between 5 ± 3 °C for up to one month, protected against dehydration.

Nutrient agar may be obtained commercially, supplemented with bromothymol blue solution and reconstituted according to manufacturer's instructions.

A8.3 *Other media*

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

9 Analytical procedure

9.1 *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 at least one of the membrane filters is likely to fall within this range. For most treated waters, 100 ml aliquots should be adequate. If, however, elevated counts are expected (for example, from raw waters) then serial dilutions using sterile quarter strength Ringer's solution may be required.

Samples should be pasteurised prior to processing by heating to 60 ± 2 °C and held for 20 ± 1 minutes, for example in a water bath. The temperature should be monitored by placing an appropriate thermometer in a bottle containing the same volume of water as the sample being treated. Commence the 20 minute heating time once the temperature has reached 60 °C. After 20 minutes remove the sample from the water bath and rapidly cool the sample by plunging the bottle, but not immersing the neck and cap, into cold water.

Before taking sub-samples and during the preparation of the dilutions, shake the sample and appropriate dilutions vigorously to ensure as homogeneous a suspension of the bacterial cells as practicable.

9.2 *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 onto the porous disc of the filter base. If a grided membrane filter is used, place grid-side upwards. 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. Record the volume of sample filtered.

Remove the funnel and carefully transfer the membrane filter to a Petri dish containing well-dried nutrient agar with bromothymol blue. 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 aerobic spore-forming bacteria can be 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, or dilution of samples, the filter base need not be sterilised unless it becomes or is suspected of being 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 for 21 ± 3 hours.

9.3 *Reading of results*

After incubation count all colonies irrespective of colour. If dilutions of the sample have been prepared select a plate for counting, preferably one in the count range of 20 to 80 colonies but otherwise one in the range 10 to 100. Colonies are usually large, typically 2 - 3 mm in diameter, and normally pale green in colour. In addition, colonies may be flat and dull in appearance with an irregular edge or raised, convex and mucoid with a smooth edge (see Figure 1).

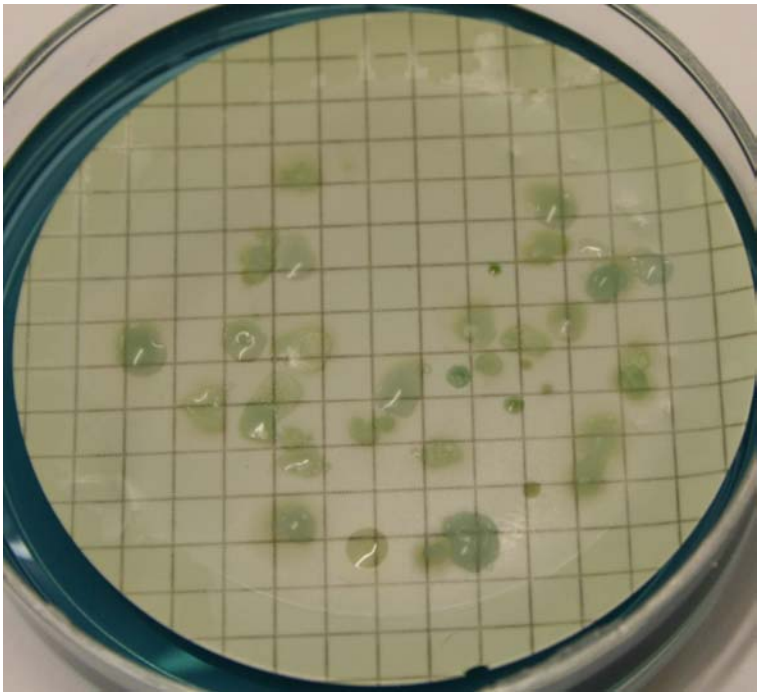
10 Calculations

The number of aerobic spore-forming bacterial colonies (ASFs) is generally quoted as the number of colonies per 100 ml. Calculate the count as follows:

$$\text{ASFs / 100 ml} = \frac{\text{Number of colonies counted on membrane filter x DF x 100}}{\text{Volume of sample filtered (ml)}}$$

Where DF is the appropriate dilution factor, if appropriate.

Figure 1 Typical aerobic spore-forming bacteria on nutrient agar supplemented with bromothymol blue



11 Expression of results

Counts for aerobic spore-forming bacteria are expressed in colony forming units per volume of sample. For most samples, the volume quoted is, typically, 100 ml.

12 Quality assurance

New batches of media should be tested by inoculating plates with spores of an appropriate spore-forming bacterium (for example a suitable strain of *Bacillus* such as *Bacillus cereus* or *Bacillus subtilis*) and incubating for 21 ± 3 hours at 37 °C. Further details are given elsewhere[(2)] in this series.

13 References

1. Using surrogates to improve plant performance. *Journal of the American Water Works Association*, E C Nieminski, W D Bellamy and L R Moss, 2000, **92**, pp67-78.

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 2002, Statutory Instrument 2002 No. 2677.

4. A simple modified membrane filtration medium for the enumeration of aerobic spore-bearing bacilli in water. *Water Research*, C A Francis, A M Lockley, D P Sartory and J Watkins, 2001, **35**, pp3758-3761.

Address for correspondence

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. In addition, if users would like to receive advanced notice of forthcoming publications please contact the Secretary on the Agency's web-page.

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