ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2002) - Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques

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 methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques

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

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 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques
Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

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.

Contents

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 is available 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

January 2002

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.

Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques

1 Introduction

The colony count of heterotrophic bacteria may be enumerated by pour or spread plate techniques using yeast extract agar[(1)] or R2A agar[(2)]. Petri dishes are typically incubated at 22 °C and 37 °C. In some cases, incubation at 30 °C can be undertaken. The most useful application of the estimation of heterotrophic bacteria populations is the detection of significant changes in trends in the bacterial content of waters. However, comparisons, between results for a particular sample, can only be made if the same method is used at all times. Details of the method should be stated in the report. Colony counts at 37 °C are useful to assess the quality of relatively unpolluted groundwaters and can provide an early indication of more serious pollution. The significance of the heterotrophic bacterial populations in water treatment and supply are described elsewhere[(3)] in this series.

2 Scope

These methods are suitable for the testing of drinking waters, including samples from all stages of treatment and distribution, and source waters.

Users wishing to employ these methods should verify their performance under their own laboratory conditions[(4)].

3 Definitions

In the context of these methods, heterotrophic bacterial colony forming units comprise bacteria capable of growth on the selected medium under the conditions specified. Whilst some yeasts and moulds are also capable of growing on the selected medium, moulds are readily distinguished. Certain yeasts, however, may produce bacterial-like colonies, and as such, would be included in any colony count.

4 Principle

Pour plates are prepared by mixing test volumes of the water sample with molten yeast extract agar or R2A agar in Petri dishes. Following incubation under the conditions specified, the number of the colonies that develop is counted.

Spread plates are prepared by spreading test volumes of the water sample onto the surface of pre-dried yeast extract agar or R2A agar in Petri dishes. Following incubation under the conditions specified, the number of the colonies that develop is counted.

5 Limitations

In the UK, the pour plate technique that employs yeast extract agar is used for compliance monitoring. Yeast extract agar is a nutrient rich medium and is known to support the growth of only a small percentage of heterotrophic bacteria present in

water. For special or unusual investigations, such as consumer complaints of taste or odour, it may be advantageous to use R2A agar. This medium is a low nutrient formulation that enhances the recovery of disinfectant-damaged organisms, and those organisms with a low nutrient requirement that are inhibited by the higher concentrations of nutrients present in yeast extract agar.

The spread plate technique may give better recoveries than the pour plate technique, as organisms in the spread plate method are not heat shocked by the addition of molten agar, as may occur in the pour plate method.

Estimations of total bacterial populations by direct counting techniques may be obtained using microscopic methods employing acridine orange[5] or 4',6-diamidino-2-phenylindole[6]. Respiring populations may be estimated using formazan-based substrates such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride[7] or 5-cyano-2,3-ditolyl-tetrazolium chloride[8].

6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[9] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[4] in this series.

7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[4] in this series. Principally, incubators (fan assisted, static temperature) 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 Incubators capable of maintaining temperatures of 22 ± 1.0 °C, and 30 ± 1.0 °C or 37 ± 1.0 °C.

7.3 Sterile 1 ml pipettes, or pipettor with sterile 1 ml pipette tips.

7.4 Colony counter, magnifying lens or image analysis colony counting system.

8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. 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.

8.1 *Yeast extract agar*

Yeast extract	3 g
Peptone	5 g
Agar	12 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients in the water and heat to dissolve. Dispense the medium (15 - 20 ml volumes) into capped tubes, or larger volumes into screw-capped bottles (for example, 400 ml into 500 ml bottles). Sterilise the medium by autoclaving at 121 °C for 15 minutes. The final pH of the medium after sterilisation should be 7.2 ± 0.2. The medium may be stored at room temperature but should be used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique may be stored at temperatures between 2 - 8 °C for up to 1 month, if protected against dehydration.

8.2 *R2A agar*

Yeast extract	500 mg
Proteose peptone No. 3 or polypeptone	500 mg
Casamino acids	500 mg
Glucose	500 mg
Soluble starch	500 mg
Dipotassium hydrogen phosphate	300 mg
Magnesium sulphate heptahydrate	50 mg
Sodium pyruvate	300 mg
Agar	12 g
Distilled, deionised or similar grade water	1 litre

Dissolve all the ingredients, except the agar, in the water. Adjust the pH to 7.2 with solid dipotassium hydrogen phosphate or potassium dihydrogen phosphate. Add the agar and heat to dissolve. Dispense the medium (in 15 - 20 ml volumes) into capped tubes, or larger volumes in screw-capped bottles (for example, 400 ml in 500 ml bottles). Sterilise by autoclaving at 121 °C for 15 minutes. The final pH should be 7.2 ± 0.2. The medium may be stored at room temperature but should be used within 1 month. Alternatively, Petri dishes containing medium to be used in the spread plate technique may be stored at temperatures between 2 - 8 °C for up to 1 month, if protected against dehydration.

8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Ringer’s solution and 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 plate lies, if possible, between 30 and 300. With some waters, it may be advantageous to plate out different volumes of sample so that the number of colonies on any one of the plates from each incubation temperature is likely to fall within this range. For treated waters, inoculate 1 ml of the sample, for polluted waters either inoculate smaller volumes, or dilute the sample with an appropriate diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) before plating out.

9.2 *Pour plate sample processing*

Heat the tube or bottle of medium and melt the medium, and keep at 45 - 48 °C. In duplicate, starting with the most dilute sample solution, pipette 1 ml of each of the diluted samples, if prepared, and 1 ml of the original sample, into separate, empty sterile Petri dishes. Pour 15 - 20 ml of molten agar into each Petri dish within 20 minutes of dispensing the 1 ml sample volumes and cover the dish with a lid. If a water bath is used to keep the medium at 45 - 48 °C, remove the water from the outside of the container, so as to avoid contamination, prior to pouring the medium into the Petri dish. Mix the sample and medium by rapid, but gentle, clockwise and anti-clockwise circular movements for approximately 10 seconds. Lift the lid slightly during mixing so that the solution does not adhere to the lid, and keep the Petri dish flat on the bench. Allow the solution to solidify and incubate in an inverted position.

9.3 *Spread plate sample processing*

If Petri dishes are to be prepared from medium contained in tubes or bottles, heat the tube or bottle of medium and the melt the medium and keep at 45 - 48 °C. If a water bath is used to keep the medium at 45 - 48 °C, remove the water from the outside of the container, so as to avoid contamination, prior to pouring the medium into the Petri dish. Pour 15 - 20 ml of molten agar into each Petri dish and allow the medium to solidify. If a prepared Petri dish is used, allow the dish to equilibrate to room temperature. The dish should be dried of excess moisture before use. In duplicate, pipette 0.1 ml of the sample, or diluted sample, onto the surface of pre-dried agar medium contained in the Petri-dish. Distribute the sample over the surface of the medium with a sterile bent glass or plastic rod. Alternatively, distribute the sample by rotating the dish whilst holding the spreader steady. Allow the inoculum to soak into the agar, and incubate in the inverted position.

9.4 *Incubation of plates*

For yeast extract agar, incubate one of the Petri-dishes at 22 °C for 68 ± 4 hours, and the duplicate Petri-dish at 37 °C for 44 ± 4 hours. Petri-dishes incubated at 37 °C may be examined after 21 ± 3 hours if necessary, for instance if an early indication of

growth is required. Samples of water in bottles or other containers[10] are incubated at 37 °C for 44 ± 4 hours.

For R2A agar, incubate one set of Petri-dishes at 22 °C for 5 - 7 days and the duplicate Petri-dish at 30 °C for 3 days.

9.5 *Reading of results*

Count the colonies on the medium containing the original sample. For Petri-dishes with very small colonies, a plate counter or magnifying lens should be used. If the count is greater than 300 then count the colonies from a Petri-dish containing diluted sample and which shows between 30 and 300 colonies. If all Petri-dishes show more than 300 colonies, record the result as greater than 300 at the highest dilution. Spreading colonies can hinder the count, and the following guidance should be used as a basis for counting a single colony forming unit:

(i) A chain of colonies that appears to be caused by the disintegration of a single clump of organisms.

(ii) A spreading growth that develops as a film at the bottom of the Petri-dish.

(iii) A colony that forms in a film of water at the edge of or over the surface of the agar.

If the colonies cannot be counted immediately after being removed from the incubator, then the Petri-dishes should be kept at 2 - 8 °C for no longer than 24 hours.

10 Calculations

Calculate the number of colonies per millilitre of sample by dividing the count by the volume (in millilitres) of the actual volume of sample pipetted into, or spread onto, the medium.

11 Expression of results

The count is quoted in terms of the number of colony forming units per millilitre. Counts greater than 300 per Petri-dish are recorded as >300 colony forming units per millilitre. Counts are increased *pro rata* if dilutions are prepared and used. When the number of colonies exceeds 300, and an attempt is made to count all colonies present, the report of the count should be accompanied with a caveat that the count is an estimate, and as such, may not reflect the true count. The term “too numerous to count” should not be used without further qualification.

12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Micrococcus luteus*). Petri-dishes should be incubated for 24 hours at 22 °C, and 30 °C or 37 °C as appropriate. Alternatively, aliquots of a

raw water, of known microbial quality, may be used. Further details are given elsewhere[4] in this series.

13 References

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4. 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.

5. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, Hobbie, J. E., Daley, R .J. & Jasper, S., 1977, **33**, 1225-1228.

6. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*, Porter, K. G. & Feig, Y. S., 1980, **25**, 943-948.

7. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Applied and Environmental Microbiology*, Zimmermann, R., Iturriaga, R. & Becker-Birck, J., 1978, **36**, 926-935.

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

9. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

10. European Union (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities*, 5.12.98, L 330/32-L 330/53.

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.

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