Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2015) – Part 4 – Methods for the isolation and enumeration of enterococci

Methods for the Examination of Waters and Associated Materials

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This booklet contains three methods for the isolation and enumeration of enterococci,

A The enumeration of enterococci by a membrane filtration technique

B The enumeration of enterococci by a defined substrate most probable number technique

C The enumeration of enterococci by a miniaturised most probable number technique

This bluebook updates and replaces section 7.4 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products. They serve only as illustrative examples of the types of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed 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, 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 (established 1972 by the Department of the Environment). At present, there are seven 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, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 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 (http://standingcommitteeofanalysts.co.uk/) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Robert Carter
Secretary
June 2015

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 resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
"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 "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

A The enumeration of enterococci by a membrane filtration technique

A1 Introduction

Tests for enterococci are used to determine the presence of faecal contamination in recreational and other waters. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water for recreational purposes. They are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of final effluents. Enterococci are important in the monitoring of shellfish water quality and for the determination of diffuse and point sources of pollution in surface waters and marine waters. Occasionally, identification of the species of enterococci or streptococci present in a sample may help to distinguish between human and animal pollution. The significance of enterococci in recreational and other waters is described elsewhere[(1)] in this series.

A2 Scope

The method is suitable for the examination of surface and marine waters, swimming pools, spa pools and hydrotherapy pools and primary and secondary wastewater effluents. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method, see method B and C.

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

A3 Definitions

Enterococci are Gram-positive cocci which form pairs or chains, possess Lancefield's Group D antigen and are catalase-negative. The organisms grow at a temperature of 44°C in the presence of bile salts and in concentrations of sodium azide that are inhibitory to coliform bacteria and most other Gram-negative bacteria.

In the context of this method, presumptive enterococci reduce (after incubation) triphenyltetrazolium chloride to insoluble red formazan to produce red, maroon or pink colonies on membrane enterococcus agar[(3)] when incubated at 37°C or 44°C. Some strains may produce colonies which are very small and/or pale in colour. Confirmation of the activity of the enzyme β-glucosidase is demonstrated by aesculin hydrolysis on bile aesculin agar (BAA) or kanamycin aesculin azide agar (KAAA) by transferring the membrane filter from membrane enterococcus agar (mEA) to one of the confirmatory media and incubating at 44°C[(4)] for up to 6 hours. Alternatively, selected colonies may be sub-cultured to BAA or KAAA and incubated at 44°C for 18 hours.

Sub-cultured isolates may also be tested for Gram stain and/or additional differentiation tests (see section A9.5). Some strains of *Streptococcus bovis* and *Streptococcus equinus*, whilst growing at 37°C on mEA may fail to grow at 44°C. In addition, some species of enterococci may fail to grow and hydrolyse aesculin when sub-cultured and grown on KAAA.

Some species display other characteristics useful for identification, such as resistant to heating at 60°C for 30 minutes, tolerance to pH 9.6, and the ability to grow in nutrient broth containing 6.5 % sodium chloride.

A4 Principle

Organisms are isolated on a membrane filter placed on the surface of an agar medium containing triphenyltetrazolium chloride. Enterococci usually produce red, maroon or pink colonies as a result of the formation of formazan. Occasionally, colonies may be colourless.

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 organisms. Where high numbers of organisms may be expected (for example, primary wastewater effluent) serial ten-fold dilutions should be made to obtain a countable number of colonies.

The medium employed recovers both faecally and environmentally derived enterococci. Other bacteria (for example, *Aerococcus viridans* and species of *Staphylococcus* and *Bacillus*) are also able to grow under the conditions described (for example 37°C). The ideal maximum number of typical and non-typical colonies that could be present on a single membrane filter from which counts are estimated is approximately 200[5], but ideally should be between 20 – 80 colonies. However, this would need to be reduced if several large colonies are present.

For this method, the enterococci may grow as very small colonies, allowing counts of up to 200 organisms per membrane filter to be estimated. If the number of colonies exceeds 200, and an attempt is made to count the target or total colonies present, the report of the results should contain a statement that the counts are estimates, and may not reflect the true number of colonies.

A6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[6] 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.

Some of the media described in this method contain sodium azide. This substance is highly toxic, and great care should be taken when these media are prepared, especially when powdered dehydrated ingredients are used. Sodium azide forms explosive compounds with metals, especially copper and lead. Waste material containing sodium azide should, therefore, be discarded into drains with care, preferably through plastic pipes. Azide compounds may be decomposed and rendered safe with excess sodium nitrite, before disposal.

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, either static temperature or temperature cycling) are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain 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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

A7.2 Incubators capable of maintaining temperatures of 37.0 ± 1.0°C and 44.0 ± 0.5°C or cycling incubators, fitted with timers, capable of attaining these temperatures.

A7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

A7.4 Sterile, membrane filters, for example white, 47 mm diameter gridded cellulose-based 0.45 µm nominal pore size.

A7.5 Smooth-tipped forceps.

A8 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 this method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of media are not within the stated range, then, before heating, they should be adjusted accordingly.

A8.1 *Membrane enterococcus agar (mEA)*

Tryptose	20 g
Yeast extract	5 g
Glucose	2 g
Dipotassium hydrogen phosphate	4 g
Sodium azide	400 mg
Agar	12 g
2,3,5-triphenyltetrazolium chloride (TTC)	
(1 % m/v aqueous solution)	10 ml
Water	1 litre

Dissolve the ingredients, except triphenyltetrazolium chloride, in the water either by steaming or bringing gently to the boil. The pH of the solution should be 7.2 ± 0.2. Filter sterilise the TTC solution through a nominal 0.2 µm membrane filter. Cool the medium to

50°C and add the sterilised TTC solution and mix well. The medium should not be stored and re-melted. Pour appropriate amounts of the complete medium directly into Petri dishes. Poured plates may be kept at a temperature of 5 ± 3°C for up to 1 month, if protected against dehydration.

Commercial formulations which already contain TTC should not be overheated when dissolving the ingredients as this may result in a deterioration of its performance due to the breakdown of TTC. If the medium is orange or pink when cooled to 50°C, then consideration should be given to discarding it.

A8.2 *Bile aesculin agar (BAA)*

Peptone	8 g
Bile salts	20 g
Iron(III) citrate	500 mg
Aesculin	1 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water and adjust the solution to a pH value of 7.1 ± 0.2. Sterilise at 121°C for 15 minutes. The final pH should be 7.1 ± 0.2. Sterile medium can be stored for up to one month at a temperature between 5 ± 3°C, if protected against dehydration.

A8.3 *Kanamycin aesculin azide agar (KAAA)*

Tryptone	20 g
Yeast extract	5 g
Sodium chloride	5 g
Sodium citrate	1 g
Aesculin	1 g
Iron(III) ammonium citrate	500 mg
Sodium azide	150 mg
Kanamycin sulphate	20 mg
Agar	12 g
Water	1 litre

Dissolve the ingredients in the water and sterilise at 121°C for 15 minutes. The final pH should be 7.0 ± 0.2. Sterile medium can be stored for up to one month at a temperature between 5 ± 3°C, if protected against dehydration.

A8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method may include nutrient agar, brain heart infusion agar, Mueller Hinton agar, MacConkey agar, nutrient broth, blood agar, bile agar, catalase reagent, Columbia blood agar base, quarter strength 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 at least one of the membrane filters is likely to fall within this range. For swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent before filtration.

A9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum. The stopcock should be in the closed position. 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, or diluted 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, maximum recovery diluent or quarter strength Ringer's solution) 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 process. 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 filtered.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing mEA. The surface of the medium should be dry and free of any surplus water. 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. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without it being placed in boiling water, provided that the smallest volume or highest dilution of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be used, or remove a funnel from the boiling water bath, allow the funnel to cool and carry out the filtration process. If funnels are to be re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples the filter base need not be sterilised unless it becomes or is suspected of being contaminated, or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. 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.

Encouraging the growth of enterococci is a balance between selectivity and sensitivity.

The growth of enterococci on mEA is better at 37°C, although some organisms resembling enterococci may also grow on this medium at this temperature. Selectivity is better at 44°C although lower counts of enterococci may be obtained, as some strains do not grow or do not grow well at 44°C. A typical approach is to incubate membrane filters from samples from swimming pools, spa pools and hydrotherapy pools at 37°C for 44 ± 4 hours hours and membrane filters from samples surface waters, saline waters and primary and secondary wastewater effluents are incubated at 37°C for 4.0 ± 0.5 hours followed by 44°C for 40 ± 4 hours.

A9.3 *Reading of results*

After incubation, count all red, maroon or pink colonies on mEA that are smooth and convex in shape (see Figure A1). These colonies are regarded as presumptive enterococci. Some types of enterococci may produce very pale colonies. Colonial size is variable. Some species of *Aerococcus* and *Staphylococcus* also grow on mEA producing red colonies, see Figure A1. Some species of *Bacillus* may produce pink colonies but these may be rough, flat and sometimes spread over the surface of the agar, see Figure A1. Other species are also able to grow, see Figure A1. The effect of particulate material on the colonial development of presumptive enterococci is shown in Figure A2.

A9.4 *Confirmation tests*

Presumptive enterococci may be confirmed by sub-culturing from mEA to BAA or KAAA and incubating at 44 ± 0.5°C for up to 18 hours, or alternatively, by transferring the membrane filter from mEA to BAA or KAAA, pre-warmed to room temperature, and incubating at 44.0 ± 0.5°C for up to 6 hours ± 5 minutes.

A9.4.1 Aesculin hydrolysis by sub-culture

Depending on the intended purpose of the analysis and the required accuracy, sub-culture from mEA a suitable number of red, maroon or pink colonies (however faint) to BAA or KAAA. 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.

From mEA, sub-culture to BAA or KAAA and incubate at 44°C for up to 18 hours. Enterococci should produce discrete colonies surrounded by a brown or black halo from aesculin hydrolysis, see Figure A3. Plates examined prior to 18 hours should be incubated up to 18 hours before final counts are determined. The development of this colour is usually evident within a few hours and should provide rapid confirmation. *Bacillus* species may produce some discoloration around the original inoculum site but should not develop discrete colonies.

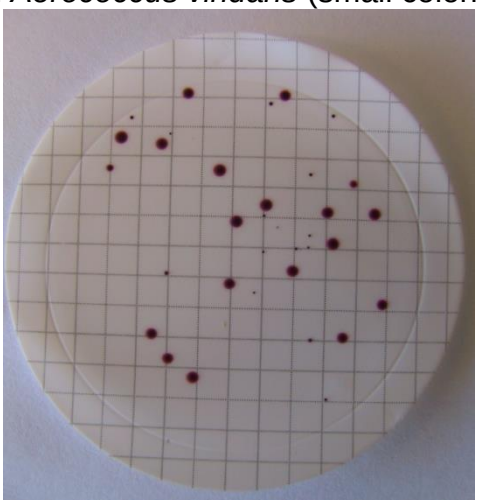
A9.4.2 Alternative aesculin hydrolysis by membrane transfer

Transfer the membrane filter from mEA carefully to a Petri dish containing BAA or KAAA, pre-warmed to room temperature, and incubate at 44.0 ± 0.5°C for up to 6 hours ± 5 minutes. The surface of the medium should be dry and free of any surplus water. 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. Colonies that are surrounded (after incubation) by a brown or black halo, resulting

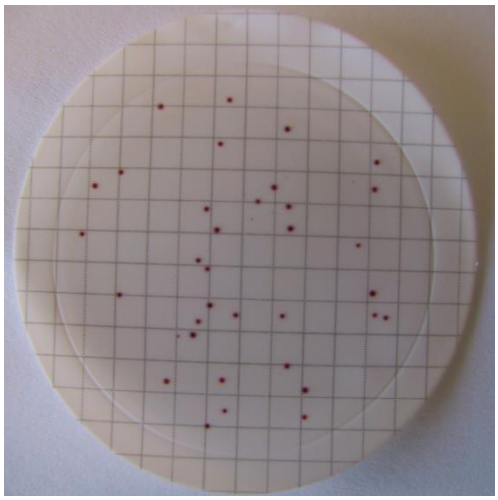
from the aesculin hydrolysis process, are regarded as confirmed enterococci (see Figure A4). The development of this halo is often evident within 2 hours and may provide rapid confirmation. However, some strains may take longer to produce the coloration, and hence the need for further incubation. In addition, where there are a substantial number of colonies on a membrane, the zone of hydrolysis from large colonies may make the interpretation or aesculin hydrolysis with adjacent smaller colonies difficult. Data on the verification of the performance of this confirmation procedure are given in Appendix 1.

Figure A1 Colonies on membrane enterococcus agar

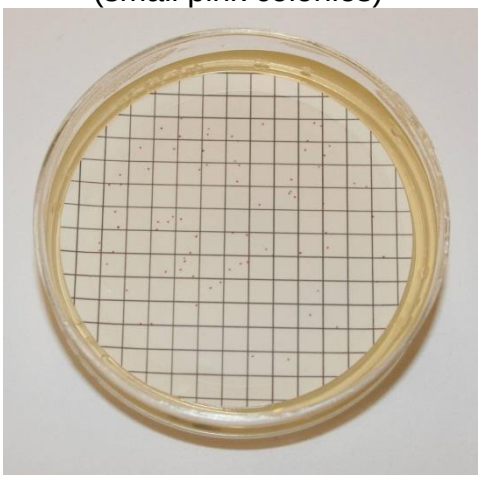
Enterococcus faecalis (large colonies) with *Aerococcus viridans* (small colonies)



Enterococcus casseliflavus



Streptococcus bovis incubated at 44°C (small pink colonies)



Aerobic spore-bearing bacilli

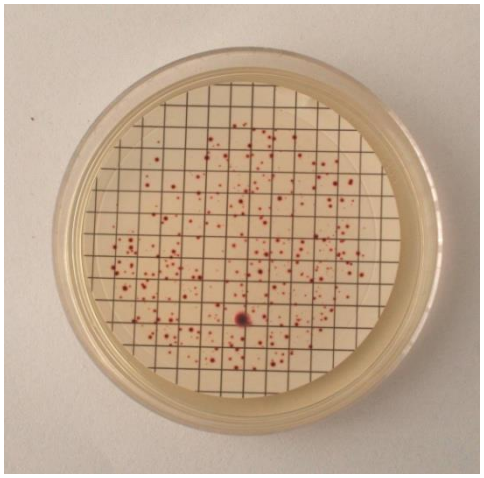


Figure A2 Effect of particulate material on colonial development of presumptive enterococci

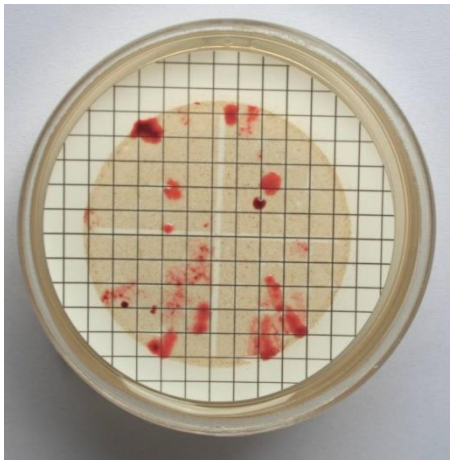


Figure A3 Colonies on kanamycin aesculin azide agar

Enterococcus faecalis *Aerococcus viridans*

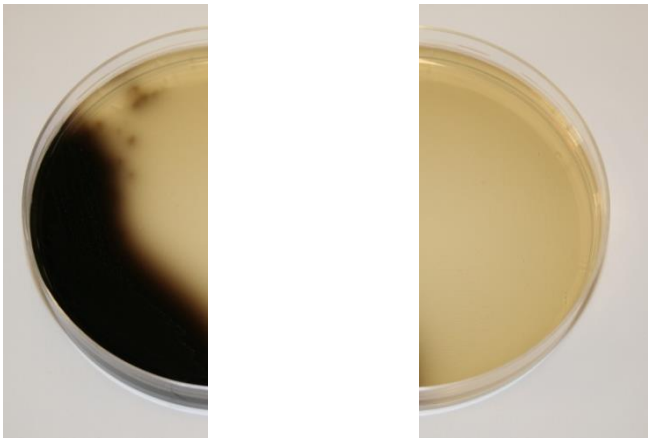
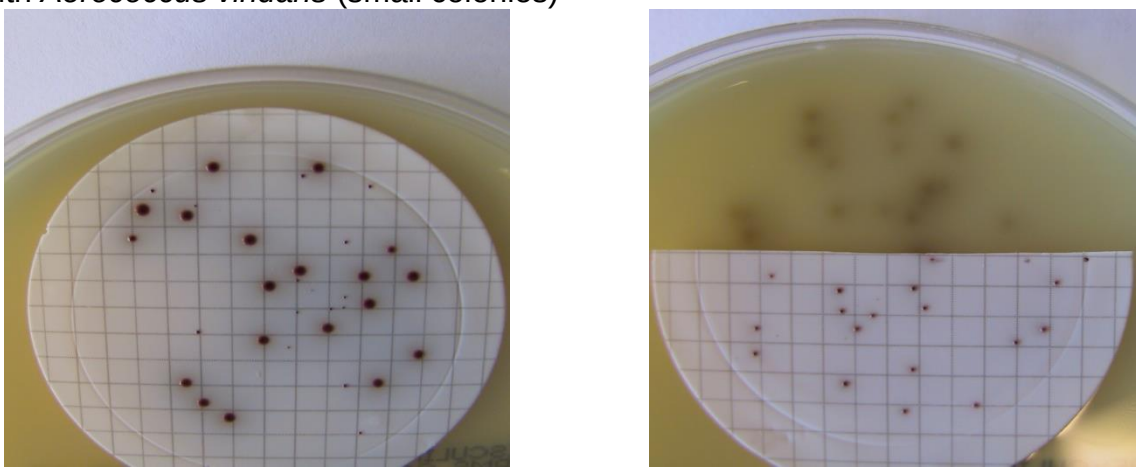


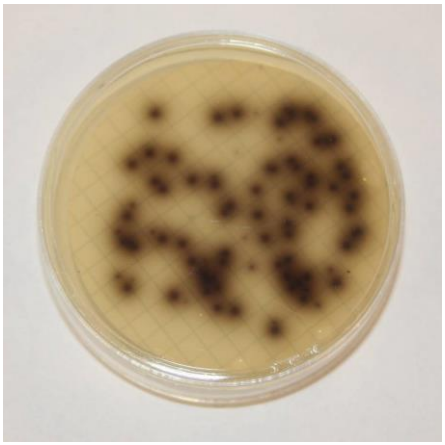
Figure A4 Colonies on bile aesculin agar following membrane transfer

Enterococcus faecalis (large colonies) with *Aerococcus viridans* (small colonies)

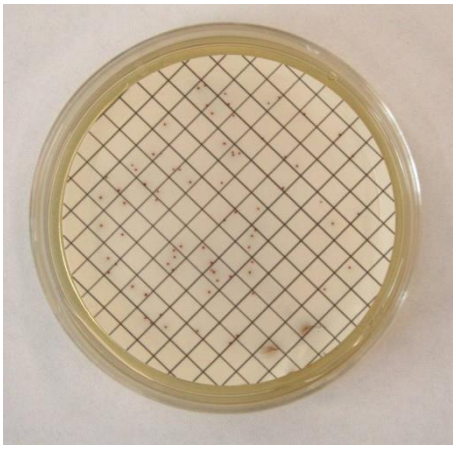
Enterococcus casseliflavus



Enterococcus faecalis aesculin hydrolysis from beneath the membrane



Streptococcus bovis showing weak aesculin hydrolysis after 6 hours at 44°C



A9.5 *Additional differentiation tests for enterococci*

Further differentiation is not usually necessary for routine water testing, but may be appropriate where particular problems are encountered. If additional tests are to be conducted, and depending on the intended purpose of the analysis and the required information, sub-culture a suitable number of aesculin-positive colonies. If the aim is to perform additional tests on the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured (if fewer than ten colonies are present) or at least ten colonies should be sub-cultured (if more than ten colonies are present). Colonies can be sub-cultured to nutrient agar although better growth is obtained on brain heart infusion agar, Mueller Hinton agar or Columbia blood agar base. Sub-cultured Petri dishes should be incubated at 37°C for 18 hours and checked to make sure that cultures are pure.

Although the possession of Lancefield's Group D antigen is referred to in the definition, serological methods of confirmation present many practical difficulties. Tolerance of 40 % bile is also characteristic of enterococci, as is a negative catalase reaction. Further tests with appropriate sub-cultures previously obtained from BAA or KAAA may be undertaken if necessary, partly as an aid to species differentiation. However, full identification depends on the demonstration of biochemical and other characteristics[7]. This may be achieved by means of one of the multi-test differential systems now available. Commercial biochemical and serological methods can be used, following appropriate verification of performance at the laboratory.

Enterococcus species are differentiated from other streptococci by their ability to grow in nutrient broth containing 6.5 % sodium chloride, and in glucose phenolphthalein broth[8] modified to pH 9.6.

A9.5.1 *Catalase test*

Enterococci are catalase-negative. For each sub-culture to be tested, emulsify some of the isolate in approximately 0.1 ml of quarter strength Ringer's solution into a small screw-capped bottle. Add approximately 0.05 ml of 5 - 6 v/v % hydrogen peroxide solution and

replace the cap. The immediate appearance of bubbles (of oxygen) indicates catalase activity.

An alternative procedure is to add approximately 0.05 ml of 5-6 v/v % hydrogen peroxide solution to colonies of a pure culture obtained from A9.5. The immediate appearance of bubbles (of oxygen) indicates catalase activity. Tests should not be carried out on media containing blood as this may result in false-positive catalase reactions. The test should preferably not be performed on a slide because of the risk of aerosol formation.

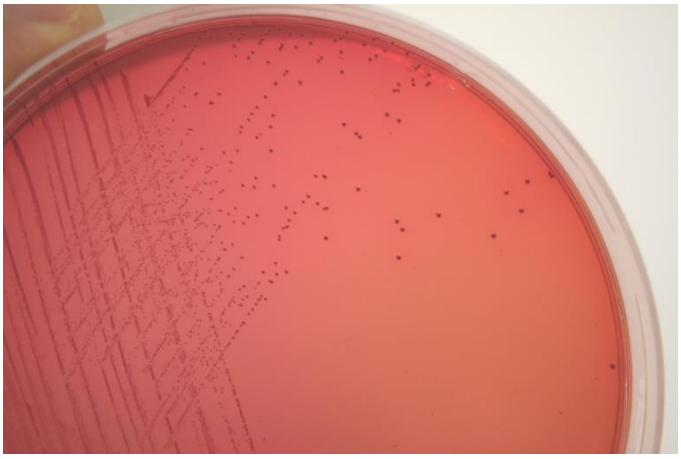
Commercial test kits for catalase testing are available and should be used in accordance with manufacturer's instructions, following appropriate performance verification at the laboratory.

On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Staphylococcus aureus* or *Escherichia coli*) and one species is known to give a negative reaction (for example, *Enterococcus faecalis*).

A9.5.2 *Bile tolerance*

Using a pure culture obtained from A9.5, sub-culture to a Petri dish or tube containing 40 % bile agar and incubate at 37°C for 24 - 48 hours. Growth on this medium indicates tolerance of bile salts. Alternatively, sub-culture to a Petri dish or tube containing MacConkey agar and incubate at 37°C for 24 - 48 hours to show growth in the presence of bile salts. Enterococci form small deep red colonies on MacConkey agar (see Figure A5). Include control tests with organisms, of which one species is known to grow in the presence of 40 % bile (for example, *Enterococcus faecalis*) and one species is known not to grow in the presence of 40 % bile (for example, *Streptococcus pneumoniae*).

Figure A5 Colonies of *Enterococcus faecalis* on MacConkey agar



A9.5.3 *Heat resistance*

Using a pure culture obtained from A9.5, transfer appropriate colonies to nutrient broth and incubate at 37°C for 21 ± 3 hours. Transfer 1 ml of the nutrient broth culture to a small test tube. Place the test tube in a water bath at 60 °C for 30 minutes. Cool the tube rapidly and incubate at 37°C for 21 ± 3 hours. Sub-culture the broth to a Petri dish containing blood

agar or other non-selective medium. Incubate overnight at 37°C and examine for growth. Include control tests with organisms, of which one species that is known to survive this heat treatment (for example, *Enterococcus faecalis*) and one species that is known not to survive (for example, *Streptococcus bovis* or *Streptococcus equinus*).

A9.5.4 *Growth at pH 9.6*

Using a pure culture obtained from A9.5, transfer a colony into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 37°C for 21 ± 3 hours. Tolerance to this broth (at pH 9.6) is demonstrated by the heavy growth of organisms and by the decolourisation of the medium from pink (red) to colourless. Include control tests with organisms, of which one species is known to grow at pH 9.6 (for example, *Enterococcus faecalis*) and one species is known not to grow at pH 9.6 (for example, *Streptococcus bovis* or *Streptococcus equinus*).

A9.5.5 *Salt tolerance*

Using a pure culture obtained from A9.5, transfer a colony from the nutrient agar and inoculate into a tube of nutrient broth containing 6.5 % of sodium chloride and incubate at 37°C for 24 - 48 hours. Examine for growth. Include control tests with organisms, of which one species is known to grow in the presence of 6.5 % salt (for example, *Enterococcus faecalis*) and one species known not to grow in the presence of 6.5 % sodium chloride (for example, *Streptococcus bovis* or *Streptococcus equinus*).

A10 Calculations

A10.1 *Presumptive enterococci*

The number of presumptive enterococci is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

$$\text{Presumptive count per 100 ml} = \frac{\text{Number of colonies on membrane filter x DF x 100}}{\text{Volume of sample filtered (ml)}}$$

where DF is the dilution factor, if appropriate.

A10.2 *Confirmed enterococci*

The number of confirmed enterococci is calculated by multiplying the number of presumptive enterococci by the proportion of the isolates that are able to hydrolyse aesculin on BAA or KAAA.

A11 Expression of results

Presumptive and confirmed enterococci are expressed in colony forming units per volume of sample. For most samples 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, *Enterococcus faecalis*) and non-target bacteria (for example,

Staphylococcus species). Petri dishes should be incubated for 44 ± 4 hours at 37°C. Appropriate method control tests should be included. Further details are given elsewhere[2] in this series.

A13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology 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*, Environment Agency.

3. Numbers of enterococci in water, sewage and faeces determined by the membrane filter technique with an improved medium, *Journal of Bacteriology*, L W Slanetz and C H Bartley, 1957, **74**, 591-595.

4. ISO 7899-2:2000 Water quality - Detection and enumeration of intestinal enterococci - Part 2 - Membrane filtration method. Geneva: International Organization for Standardization.

5. ISO 8199:2005 - Water quality - General guidance on the enumeration of micro-organisms by culture, Geneva: International Organization for Standardization

6. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

7. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, G I Barrow and R K A Feltham), London, Cambridge University Press, 1993.

8. Growth of streptococci in a glucose phenolphthalein broth, *Journal of General Microbiology*, P H Clarke, 1953, **9**, 350-352.

B The enumeration of enterococci by a defined substrate most probable number technique

This method has not been subjected to widespread use within the UK or verification of 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. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

B1 Introduction

Tests for enterococci are used to determine the presence of faecal contamination in recreational and other waters. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water for recreational purposes. They are used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of final effluents. Enterococci are important in the monitoring of shellfish water quality and for the determination of diffuse and point sources of pollution in surface freshwaters and sea waters. Occasionally, identification of the species of enterococci or streptococci present in a sample may help to distinguish between human and animal pollution. The significance of enterococci in recreational and other waters is described elsewhere[1] in this series.

B2 Scope

This method comprises a most probable number (MPN) technique and is suitable for the examination of fresh and saline surface waters, swimming pools, spa and hydrotherapy pools and primary and secondary wastewater effluents, and water samples with high turbidities.

Whilst details of a specific commercial product are described in this document, this does not constitute an endorsement of the product but serves only as an illustrative example of the types of products available. Details of this method are included for information purposes only, as an example of the defined substrate techniques that are available.

Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions[2].

B3 Definitions

Defined substrate media are chemically defined formulations containing substrates for the specific detection of diagnostic enzymes associated with a particular group of organisms.

In the context of this method, organisms which produce β-glucosidase, as demonstrated by the production of a blue fluorescence (under long wavelength ultra-violet illumination) through the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucoside present in a defined substrate medium, are regarded as enterococci. This method is reported to be highly specific for enterococci and confirmation tests are not usually required.

B4 Principle

Organisms are grown in a defined liquid medium containing a substrate for the specific detection of the enzyme β-glucosidase. The dehydrated medium is dissolved in 100 ml of sample, or 100 ml of diluted sample, which is then added to a 51-well reaction pouch. This is then sealed and incubated at 41°C ± 0.5°C for 24 - 28 hours. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth in the medium after incubation, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see Appendix B1. Alternatively, for expected higher counts a 97-well reaction pouch can be employed.

B5 Limitations

This method is suitable for most types of aqueous samples, except those with high turbidities, which may mask or inhibit fluorescence development.

This method is designed to enumerate the most important strains of intestinal enterococci (*Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae*) and thus other faecal or environmental enterococci may not be recovered.

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.

When ultra-violet lamps are used gloves and either goggles or a face shield suitable for use with appropriate ultra-violet-emitting sources should be worn.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, fan assisted incubators are required. An example of the methodology for this type of method is presented and is based upon a commercially available system. Some of the equipment listed is specific to this system and alternative systems may be available for which other equipment may be required. Other items include:

B7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain 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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

B7.2 Incubator capable of maintaining a temperature of 41.0 ± 0.5°C.

B7.3 Sterile 100 ml plastic bottles containing anti-foaming agent as supplied by the manufacturer of the test system or suitable equivalent.

B7.4 MPN reaction pouches as supplied by the manufacturer (for example, a 51-well system if low counts are expected or a 97-well system if high counts are expected) and associated heat-sealing equipment.

B7.5 Ultra-violet long wavelength (365 - 366 nm) lamp, and viewer.

B7.6 Fluorescence comparator as supplied by the manufacturer.

B8 Media and reagents

Commercial formulations of these media and reagents may be available. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method[2].

B8.1 *Enterolert-E medium*[4]

The medium is a commercially available formulation provided in sachets and is suitable for single samples. The medium is a chemically defined formulation with minimal nutrients and a substrate for the specific detection of the enzyme β-glucosidase. For MPN counts, the medium can be used in conjunction with Quanti-Tray® reaction pouches.

B9 Analytical procedure

B9.1 *Sample preparation*

The volume, or dilution, of samples should be chosen so that not all the wells show a positive response. For swimming pool and spa pool waters 100 ml of sample will generally be appropriate whilst for contaminated waters, appropriate dilutions should be prepared, and 100 ml of diluted sample used. When preparing dilutions use sterile distilled, deionised or similar grade water. Buffered solutions should not be used as they may adversely affect the performance of the method. For freshwater and marine recreational waters the sample should be diluted 1 in 10 sterile distilled, deionised or similar grade water prior to analysis or preparation of further dilutions.

B9.2 *Sample processing*

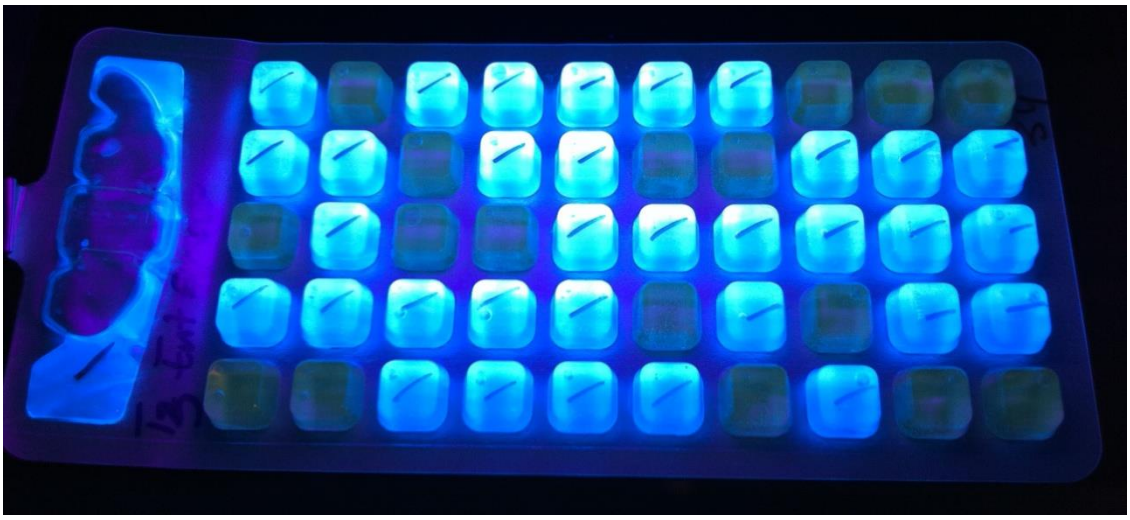
The sample or appropriate dilution (100 ml) is decanted into a sterile bottle containing an anti-foam agent. Following the manufacturer's instructions, the contents of one sachet of medium is then aseptically added. After capping the bottle, the contents are gently agitated to ensure dissolution of the medium and then the bottle is left to stand for 10 minutes or until completely dissolved and dispersal of any air bubbles completed. The contents of the bottle are then added to the MPN reaction pouch, which is then sealed in the apparatus provided by the manufacturer to produce a 51-well reaction pouch. If higher counts are expected a 97-well MPN reaction pouch can be used. Prolonged exposure of the inoculated reaction pouches to direct sunlight should be avoided as this may result in hydrolysis of the specific substrates causing false-positive reactions. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

Sealed MPN reaction pouches are then incubated, ‘well-side’ down, at 41°C for not less than 24 hours and not more than 28 hours. However, if the sample contains sediment or other particles the reaction pouches should be incubated ‘well-side’ up to avoid accumulation of material on the well wall that may interfere with the reading of positive wells.

B9.3 *Reading of results*

After incubation, the pouch is examined under a long wavelength ultra-violet lamp and the number of wells that produce a blue-white fluorescence (see Figure B1) of sufficient intensity compared against the manufacturer’s comparator, is recorded. If the pouch is examined before the completion of 24 hours incubation and this examination reveals borderline responses, then it should be returned to the incubator for the remaining incubation period. After 28 hours incubation, the pouch is removed and re-examined as before.

Figure B1 Example of 51-well MPN reaction pouch with a defined substrate medium with 34 wells showing presence of enterococci



B9.4 *Confirmation tests*

This method is reported to be highly specific for enterococci. Hence, confirmation tests are not usually required. Should there be any doubt as to the type of organism and response detected, then wells showing a positive response should be sub-cultured and confirmatory tests undertaken (see section A9.4).

B10 Calculations

B10.1 *Confirmed enterococci*

The MPN of enterococci is determined by reference to appropriate probability tables, see for example Appendix B1 for a 51-well reaction pouch. This is derived from the number of wells showing blue-white fluorescence. For example, if there are 34 wells showing fluorescence in a 51-well reaction pouch (as in Figure B1) then from Appendix B1 the MPN of enterococci is 56 per 100 ml of sample, or diluted sample, examined. Any dilution needs to be taken into account.

B11 Expression of results

Confirmed enterococci counts are expressed as MPN counts per volume of sample after adjustment for any dilution. For most samples, the volume is typically 100 ml.

B12 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *Enterococcus faecalis*) and non-target bacteria (for example, *Escherichia coli*). Pouches should be incubated for 24 - 28 hours at 41°C. Further details are given elsewhere[2] in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology 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*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9LW.

Appendix B1 MPN (and 95% confidence intervals) per 100 ml for a 51-well defined substrate medium reaction pouch

Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits	Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits
0	0	0-4	26	36	25-54
1	1	0-6	27	38	26-57
2	2	1-7	28	41	28-60
3	3	1-9	29	43	30-63
4	4	2-11	30	45	32-66
5	5	2-12	31	48	33-69
6	6	3-14	32	50	35-73
7	8	4-16	33	53	38-76
8	9	5-17	34	56	40-80
9	10	5-19	35	59	42-84
10	11	6-21	36	62	45-89
11	12	7-22	37	66	47-94
12	14	8-24	38	70	50-99
13	15	9-26	39	74	53-105
14	16	10-28	40	78	56-111
15	18	11-29	41	83	60-118
16	19	12-31	42	89	64-126
17	21	13-33	43	95	68-135
18	22	14-35	44	101	73-146
19	24	15-37	45	109	79-159
20	25	17-39	46	118	85-175
21	27	18-42	47	130	93-195
22	29	19-44	48	145	102-224
23	31	20-46	49	165	115-272
24	32	22-49	50	201	136-388
25	34	23-51	51	>201	

C The enumeration of enterococci by a miniaturised most probable number technique

This method has not been subjected to widespread use within the UK or verification of 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. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

C1 Introduction

Tests for enterococci are used to determine the presence of faecal contamination in recreational and other waters. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water for recreational purposes. They are used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of final effluents. Enterococci are important in the monitoring of shellfish water quality and for the determination of diffuse and point sources of pollution in surface freshwaters and sea waters. Occasionally, identification of the species of enterococci or streptococci present in a sample may help to distinguish between human and animal pollution. The significance of enterococci in recreational and other waters is described elsewhere[(1)] in this series.

C2 Scope

The method is suitable for the examination of fresh and saline surface waters, sediments including sand, and primary and secondary wastewater effluents, and water samples with higher turbidities. The method may only be applicable to samples containing enterococci in excess of 15 colony forming units in the volume examined.

Whilst details of a specific commercial product are described in this document, this does not constitute an endorsement of the product but serves only as an illustrative example of the type of product available. Details of this method are included for information purposes only, as an example of micro-titre plate methods that are available.

This method is based on ISO 7899-1:1998[(2)]. Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions[(3)].

C3 Definitions

In the context of this method, organisms which grow in the presence of thallium acetate and which produce β-glucosidase, as demonstrated by the production of a blue-white fluorescence (under ultra-violet illumination at 366 nm) through the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucoside (MUD) in a selective medium at 44°C in 36 - 72 hours, are regarded as enterococci.

C4 Principle

A micro-titre plate, comprising 96 wells, is used whereby portions of the diluted sample are inoculated into several rows of wells, each containing dehydrated culture medium. The plates are then incubated for between 36 - 72 hours at 44°C. If present, enterococci are indicated in each well by a blue fluorescence as seen under ultra-violet illumination following hydrolysis of 4-methylumbelliferyl-β-D-glucoside (MUD). The most probable number of organisms in 100 ml of sample is then estimated from appropriate probability tables.

C5 Limitations

This method is not suitable for samples where enterococci are likely to be absent, or present in low numbers.

This method is designed to enumerate the most important strains of intestinal enterococci (*Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae*) and thus other faecal or environmental enterococci may not be recovered.

C6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

N,N-dimethylformamide and thallium acetate, which are components of one of the media, are toxic and carcinogenic. Inhalation, contact with skin or ingestion of these substances should be avoided, and should be handled within a fume cupboard.

When ultraviolet lamps are used, gloves and either goggles or a face shield suitable for use with appropriate ultraviolet-emitting sources should be worn.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally, fan assisted incubators are required. Other items include:

C7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain 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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

C7.2 Incubator capable of maintaining a temperature of 44.0 ± 0.5°C.

C7.3 Tunnel drier, fume cabinet or vertical laminar air flow cabinet.

C7.4 Ultra-violet long wavelength (365 - 366 nm) lamp and viewer.

C7.5 8-channel multi-pipette, with suitable sterile tips, adjustable or pre-set, capable of dispensing 200 µl of diluted sample into each well (or other equivalent system).

C7.6 Sterile 96-well micro-titre plates and sterile adhesive covering strips for sealing the wells.

C8 Media and reagents

Commercial formulations of some of these media and reagents are available. The performance of all media and reagents should be verified prior to their use. Variations in the preparation and storage of media should also be verified[3]. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. Water should be distilled, deionised or of similar grade quality.

C8.1 *MUD/SF medium*[2]

SOLUTION 1

Tryptone	40 g
Potassium dihydrogen phosphate	10 g
D(+)-galactose	2 g
Polyoxyethylenesorbitan monooleate (for example Tween® 80)	1.5 ml
Water	900 ml

Stir the ingredients in the water with gentle heating and then bring to the boil to dissolve completely. Allow the solution to cool.

SOLUTION 2

Sodium hydrogen carbonate	4 g
Nalidixic acid	250 mg
Water	50 ml

Dissolve the ingredients in the water with gentle heating and allow the solution to cool.

SOLUTION 3

Thallium(I) acetate	2 g
2,3,5-triphenyltetrazolium chloride	1 g
Water	50 ml

Dissolve the ingredients in the water with gentle heating in a fume cabinet and allow the solution to cool.

SOLUTION 4

MUD (4-methylumbelliferyl-β-D-glucoside)	150 mg
N,N dimethylformamide	2 ml

Add the whole of solutions 2, 3 and 4 to solution 1. Adjust the pH to 7.5 ± 0.2. Sterilise by membrane filtration (0.2 µm pore size). Dispense 100 µl of the sterile solution into each well contained in a 96-well micro-titre plate. Immediately dehydrate the medium using a tunnel drier, fume cabinet or laminar air flow cabinet (B7.3). Dried micro-titre

plates may be stored in the dark at 5 ± 3°C for up to one month in appropriate sterile packaging, prior to use. Prepared micro-titre plates are available commercially and should be used and stored according to manufacturer's instructions.

C8.2 *Special diluent*

Synthetic sea salt	22.5 g
Bromophenol blue (0.04 % m/v in 50 % aqueous ethanol)	10 ml
Water	1 litre

Dissolve the ingredients in the water and sterilise by autoclaving at 121°C for 15 - 20 minutes.

Special diluent may be stored at room temperature for up to two months.

Alternatively, a special diluent can be prepared as follows:

SOLUTION 5

Calcium chloride dihydrate	83.6 g
Potassium chloride	43.5 g
Strontium chloride hexahydrate	0.07 g
Water	1 litre

Heat the ingredients in the water to approximately 50°C to dissolve.

SOLUTION 6

Sodium hydrogen carbonate	15.15 g
Sodium borate	3 g
Water	1 litre

Heat the ingredients in the water to approximately 50°C to dissolve.

SOLUTION 7

Magnesium sulphate heptahydrate	190 g
Magnesium chloride	147 g
Water	1 litre

Heat the ingredients in the water to approximately 50°C to dissolve.

Add 10 ml of solution 5, 10 ml of solution 6 and 20 ml of solution 7 to 950 ml of water. Add 14.9 g of sodium chloride, dissolve and adjust the pH to 7.5 ± 0.2. Add 10 ml of a 0.04 % m/v solution of bromophenol blue in 50 % aqueous ethanol and mix well. Dispense in suitable volumes in containers and sterilise by autoclaving at 121°C for 15 - 20 minutes.

C8.3 *Other media*

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

C9 Analytical procedure

C9.1 *Volumes of sample for inoculation*

Different dilutions of the same sample are inoculated into the wells of the micro-titre plate. The strength of the diluted samples will depend on the likely degree of faecal contamination of the sample.

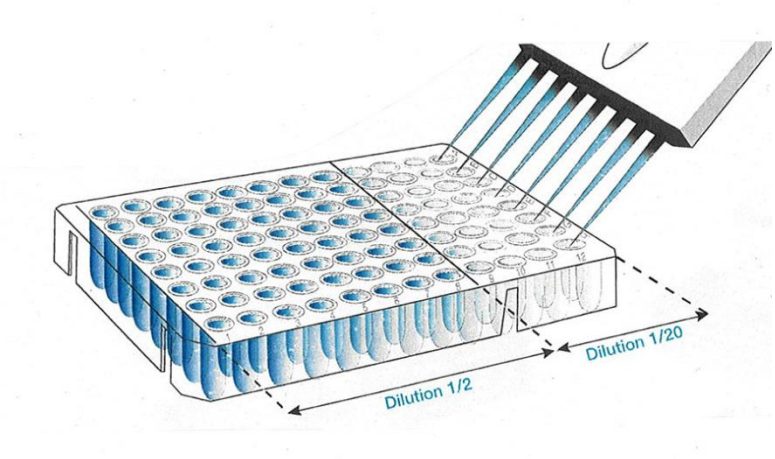
For saline waters, dilutions should be prepared using the special diluent (C8.2). For surface waters and wastewaters, dilutions can be prepared in quarter strength Ringer's solution or maximum recovery diluent.

For example, for bathing waters and moderately polluted surface waters prepare two dilutions of the sample, i.e.

1) dilute 20 ml of sample to 40 ml, i.e. a 1 in 2 dilution;
2) dilute 2 ml of sample to 40 ml, i.e. a 1 in 20 dilution.

Dispense 200 µl of the first diluted sample to each of the first 64 wells. See Figure C1. Identify each well. Dispense 200 µl of the second diluted sample to each of the remaining 32 wells. See Figure C1. Identify each well. This should enable counts to be obtained in the range 15 – 35000 enterococci per 100 ml of sample, i.e. a minimum of 15 up to 3.5 X 10^4.

Figure C1 Typical micro-titre plate with series of dilutions



For polluted fresh surface waters prepare 4 dilutions, i.e.

1) dilute 20 ml of sample to 40 ml, i.e. a 1 in 2 dilution.

From this solution prepare 3 further serial dilutions, namely

2) dilute 2 ml of solution 1) to 20 ml, i.e. a 1 in 20 dilution;
3) dilute 2 ml of solution 2) to 20 ml, i.e. a 1 in 200 dilution;
4) dilute 2 ml of solution 3) to 20 ml, i.e. a 1 in 2000 dilution.

Dispense 200 µl of each diluted sample to each of 24 wells. Identify each well. This should enable counts to be obtained in the range 40 - 3200000 enterococci per 100 ml of sample, i.e. a minimum of 40 up to 3.2 X 10^6.

For wastewater, prepare 6 dilutions, i.e.

1) dilute 20 ml of sample to 40 ml; i.e. a 1 in 2 dilution.

From this solution prepare 5 further serial dilutions, namely

2) dilute 2 ml of solution 1) to 20 ml, i.e. a 1 in 20 dilution;
3) dilute 2 ml of solution 2) to 20 ml, i.e. a 1 in 200 dilution;
4) dilute 2 ml of solution 3) to 20 ml, i.e. a 1 in 2000 dilution;
5) dilute 2 ml of solution 4) to 20 ml, i.e. a 1 in 20000 dilution;
6) dilute 2 ml of solution 5) to 20 ml, i.e. a 1 in 200000 dilution.

Dispense 200 µl of each diluted sample to each of 16 wells. Identify each well. This should enable counts to be obtained in the range 60 - 670000000 enterococci per 100 ml of sample, i.e. a minimum of 60 up to 6.7 X 10^8.

For all types of samples and in all cases, a sufficient number of diluted samples should be prepared and added to wells of the micro-titre plate. Following incubation, for at least one of the diluted samples within the series, some wells within the micro-titre plate should exhibit growth within the medium and some wells should show no growth within the medium.

C9.2 *Sample processing*

For each prepared diluted sample, label a sterile 90 mm diameter Petri dish. Decant an appropriate volume (typically 15 - 20 ml) of each diluted sample into their respective labelled Petri dishes.

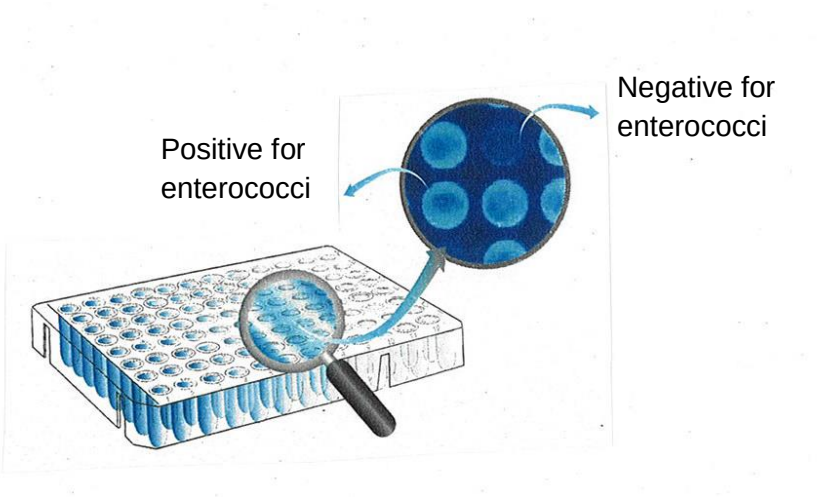
Use the multi-channel pipettor, set to deliver 200 µl from each channel, and eight sterile pipette tips to inoculate each diluted sample into the required number of wells in the micro-titre plate according to the type of sample and inoculation pattern described in section C9.1. After the wells of medium have been inoculated with the diluted samples, each well is covered with disposable sterile adhesive tape (C7.6).

Incubate the plates at 44.0 $\pm$ 0.5°C for not less than 36 hours and not more than 72 hours.

C9.3 *Reading of results*

After incubation, examine the wells under ultraviolet light. Wells showing a blue-white fluorescence indicate the presence of enterococci and are considered positive (see Figure C2). The number of positive wells for each diluted sample is recorded.

Figure C2 Example of MPN micro-titre plate with a defined substrate medium with fluorescing wells showing presence of enterococci



C9.4 *Confirmation tests*

This method is reported to be highly specific for enterococci (as demonstrated by blue-white fluorescence in the wells). Hence, confirmation tests are not usually required. Should there be any doubt as to the type of organism and response detected, then wells showing a positive response should be sub-cultured and confirmatory tests undertaken (see Section A9.4).

C10 Calculations

C10.1 *Confirmed enterococci*

The MPN of enterococci in the sample is determined by a basic computer programme available in Annexes A and B of ISO 7899-1:1998[(2)]. Statistical tables are supplied by the manufacturer[(5)] of the product and an example of how an MPN is derived is described below.

For a two-dilution series, the MPN of enterococci in the sample is determined using the tables in Appendix C1. Similar tables are available for 4- and 6-dilution series. A basic computer programme is available in Annexes A and B of ISO 7899-1:1998[(2)].

The number of wells exhibiting growth (i.e. positive wells) and number of wells inoculated in the micro-titre plate are used to generate a MPN.

Example of a two dilution series on a sea water sample:

Dilution	Number of positive wells	Number of wells inoculated
1/2	32	64
1/20	5	32

From these numbers and using the tables for two dilutions (see Appendix C1) then this corresponds to an MPN of 756 enterococci / 100 ml.

C11 Expression of results

Confirmed enterococci counts are expressed as MPN counts per volume of sample. For most samples, the volume is typically 100 ml.

C12 Quality assurance

The quality assurance in the preparation of the trays is described in ISO 7899-1:1998[(2)]. In addition, the standard also gives details of the preparation of calibration micro-titre plates. New batches of media should be tested by inoculating a suspension containing a known number of organisms of the appropriate reference bacteria (for example *Enterococcus faecalis* and non-target bacteria (for example, *E. coli*). In addition, a suspension containing a known number of organisms of these reference organisms should be included with each batch of tests.

C13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. ISO 7899-1:1998 - Water quality - detection and enumeration of intestinal enterococci bacteria in surface and wastewater - Part 1 - Miniaturised method (most probable number) by inoculation in liquid medium. Geneva: International Organization for Standardization.

3. 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*, Environment Agency.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

5. Bio-Rad Laboratories Limited, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX.

Appendix C1 MPN tables based on the micro-titre technique

Based on a two-dilution series the following tables are used to determine the MPN of organisms in 100 ml of sample. With each MPN there would be associated lower and upper limit values. Similar tables are available for 4- and 6-dilution series.

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
0	1	15	9	0	144	16	0	272
0	2	30	9	1	160	16	1	289
0	3	45	9	2	176	16	2	307
0	4	60	9	3	192	16	3	324
			9	4	209	16	4	342
1	0	15	9	5	225	16	5	359
1	1	30	9	6	241	16	6	377
1	2	45				16	7	394
1	3	60	10	0	161			
1	4	75	10	1	177	17	0	292
1	5	90	10	2	194	17	1	309
			10	3	210	17	2	327
2	0	30	10	4	226	17	3	344
2	1	45	10	5	243	17	4	362
2	2	61	10	6	259	17	5	380
2	3	76				17	6	398
2	4	91	11	0	179	17	7	416
2	5	106	11	1	195	17	8	433
			11	2	212			
3	0	46	11	3	228	18	0	312
3	1	61	11	4	245	18	1	330
3	2	76	11	5	261	18	2	347
3	3	92	11	6	278	18	3	365
3	4	107	11	7	295	18	4	383
3	5	122				18	5	401
			12	0	197	18	6	419
4	0	61	12	1	213	18	7	437
4	1	77	12	2	230	18	8	455
4	2	92	12	3	247			
4	3	108	12	4	263	19	0	332
4	4	123	12	5	280	19	1	350
4	5	139	12	6	297	19	2	368
			12	7	314	19	3	386
5	0	77				19	4	405
5	1	93	13	0	215	19	5	423
5	2	108	13	1	232	19	6	441
5	3	124	13	2	249	19	7	459
5	4	140	13	3	266	19	8	478
5	5	155	13	4	282			
5	6	171	13	5	299	20	0	353
			13	6	316	20	1	371
6	0	94	13	7	333	20	2	390
6	1	109				20	3	408
6	2	125	14	0	234	20	4	426
6	3	141	14	1	251	20	5	445
6	4	156	14	2	268	20	6	463
6	5	172	14	3	285	20	7	482
6	6	188	14	4	302	20	8	500
			14	5	319			
7	0	110	14	6	336	21	0	375
7	1	126	14	7	353	21	1	393
7	2	142				21	2	412
7	3	158	15	0	253	21	3	430

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
7	4	174	15	1	270	21	4	449
7	5	189	15	2	287	21	5	467
7	6	205	15	3	304	21	6	486
			15	4	322	21	7	505
8	0	127	15	5	339	21	8	524
8	1	143	15	6	356			
8	2	159	15	7	374			
8	3	175						
8	4	191						
8	5	207						
8	6	223						
22	0	397	28	0	539	34	0	705
22	1	415	28	1	559	34	1	728
22	2	434	28	2	580	34	2	750
22	3	453	28	3	600	34	3	773
22	4	472	28	4	621	34	4	796
22	5	490	28	5	641	34	5	818
22	6	509	28	6	662	34	6	841
22	7	528	28	7	683	34	7	865
22	8	547	28	8	704	34	8	888
			28	9	725	34	9	911
23	0	419	28	10	746	34	10	935
23	1	438				34	11	958
23	2	457	29	0	565			
23	3	476	29	1	585	35	0	736
23	4	495	29	2	606	35	1	759
23	5	514	29	3	627	35	2	782
23	6	533	29	4	648	35	3	805
23	7	553	29	5	669	35	4	828
23	8	572	29	6	690	35	5	851
23	9	591	29	7	711	35	6	875
			29	8	732	35	7	898
24	0	442	29	9	753	35	8	922
24	1	461	29	10	775	35	9	946
24	2	480				35	10	970
24	3	500	30	0	591	35	11	994
24	4	519	30	1	612			
24	5	538	30	2	633	36	0	767
24	6	558	30	3	654	36	1	791
24	7	577	30	4	676	36	2	814
24	8	597	30	5	697	36	3	838
24	9	617	30	6	718	36	4	861
			30	7	740	36	5	885
25	0	465	30	8	761	36	6	909
25	1	485	30	9	783	36	7	933
25	2	504	30	10	805	36	8	957
25	3	524				36	9	982
25	4	543	31	0	619	36	10	1006
25	5	563	31	1	640	36	11	1031
25	6	583	31	2	661			
25	7	603	31	3	683	37	0	800
25	8	623	31	4	704	37	1	824
25	9	643	31	5	726	37	2	848
			31	6	748	37	3	872
26	0	489	31	7	770	37	4	896
26	1	509	31	8	792	37	5	920
26	2	529	31	9	814	37	6	945
26	3	549	31	10	836	37	7	969
26	4	568				37	8	994

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
26	5	588	32	0	647	37	9	1019
26	6	609	32	1	668	37	10	1044
26	7	629	32	2	690	37	11	1069
26	8	649	32	3	712			
26	9	669	32	4	734	38	0	834
			32	5	756	38	1	858
27	0	514	32	6	778	38	2	882
27	1	534	32	7	800	38	3	907
27	2	554	32	8	823	38	4	931
27	3	574	32	9	845	38	5	956
27	4	594	32	10	868	38	6	981
27	5	615				38	7	1006
27	6	635	33	0	675	38	8	1032
27	7	655	33	1	697	38	9	1057
27	8	676	33	2	720	38	10	1083
27	9	696	33	3	742	38	11	1109
			33	4	764	38	12	1135
			33	5	787			
			33	6	809			
			33	7	832			
			33	8	855			
			33	9	878			
			33	10	901			
39	0	868	44	0	1064	48	0	1253
39	1	893	44	1	1092	48	1	1285
39	2	918	44	2	1120	48	2	1317
39	3	943	44	3	1148	48	3	1349
39	4	969	44	4	1177	48	4	1382
39	5	994	44	5	1206	48	5	1415
39	6	1020	44	6	1235	48	6	1448
39	7	1045	44	7	1265	48	7	1482
39	8	1071	44	8	1294	48	8	1516
39	9	1097	44	9	1324	48	9	1550
39	10	1123	44	10	1354	48	10	1585
39	11	1150	44	11	1385	48	11	1620
39	12	1176	44	12	1416	48	12	1655
			44	13	1446	48	13	1691
40	0	904				48	14	1727
40	1	930	45	0	1108			
40	2	955	45	1	1136	49	0	1306
40	3	981	45	2	1166	49	1	1339
40	4	1007	45	3	1195	49	2	1372
40	5	1033	45	4	1225	49	3	1406
40	6	1059	45	5	1254	49	4	1440
40	7	1086	45	6	1285	49	5	1474
40	8	1112	45	7	1315	49	6	1509
40	9	1139	45	8	1345	49	7	1544
40	10	1166	45	9	1376	49	8	1579
40	11	1193	45	10	1407	49	9	1615
40	12	1220	45	11	1439	49	10	1651
			45	12	1471	49	11	1688
41	0	942	45	13	1503	49	12	1725
41	1	968				49	13	1762
41	2	994	46	0	1154	49	14	1800
41	3	1020	46	1	1183	49	15	1838
41	4	1047	46	2	1213			
41	5	1074	46	3	1244	50	0	1363
41	6	1100	46	4	1274	50	1	1397

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
41	7	1128	46	5	1305	50	2	1431
41	8	1155	46	6	1336	50	3	1466
41	9	1182	46	7	1368	50	4	1502
41	10	1210	46	8	1399	50	5	1537
41	11	1238	46	9	1431	50	6	1573
41	12	1266	46	10	1463	50	7	1610
			46	11	1496	50	8	1647
42	0	981	46	12	1529	50	9	1684
42	1	1007	46	13	1562	50	10	1722
42	2	1034	46	14	1595	50	11	1760
42	3	1061				50	12	1799
42	4	1089	47	0	1202	50	13	1838
42	5	1116	47	1	1233	50	14	1878
42	6	1143	47	2	1264	50	15	1918
42	7	1171	47	3	1295			
42	8	1199	47	4	1327	51	0	1423
42	9	1227	47	5	1358	51	1	1458
42	10	1256	47	6	1391	51	2	1494
42	11	1284	47	7	1423	51	3	1531
42	12	1313	47	8	1456	51	4	1567
			47	9	1489	51	5	1605
43	0	1021	47	10	1522	51	6	1642
43	1	1049	47	11	1556	51	7	1681
43	2	1076	47	12	1590	51	8	1719
43	3	1104	47	13	1625	51	9	1758
43	4	1132	47	14	1659	51	10	1798
43	5	1160				51	11	1838
43	6	1188				51	12	1879
43	7	1217				51	13	1920
43	8	1246				51	14	1962
43	9	1275				51	15	2004
43	10	1304						
43	11	1333						
43	12	1363						
43	13	1393						
52	0	1486	55	0	1706	58	0	1988
52	1	1523	55	1	1749	58	1	2041
52	2	1561	55	2	1793	58	2	2095
52	3	1599	55	3	1838	58	3	2150
52	4	1638	55	4	1883	58	4	2206
52	5	1677	55	5	1929	58	5	2263
52	6	1716	55	6	1976	58	6	2322
52	7	1756	55	7	2023	58	7	2383
52	8	1797	55	8	2072	58	8	2444
52	9	1838	55	9	2121	58	9	2508
52	10	1880	55	10	2172	58	10	2573
52	11	1922	55	11	2223	58	11	2640
52	12	1965	55	12	2275	58	12	2708
52	13	2009	55	13	2328	58	13	2779
52	14	2053	55	14	2382	58	14	2851
52	15	2098	55	15	2438	58	15	2926
52	16	2143	55	16	2494	58	16	3002
			55	17	2551	58	17	3082
53	0	1554				58	18	3163
53	1	1593	56	0	1792	58	19	3247
53	2	1633	56	1	1838			
53	3	1673	56	2	1884	59	0	2104

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
53	4	1713	56	3	1931	59	1	2161
53	5	1754	56	4	1980	59	2	2219
53	6	1796	56	5	2029	59	3	2279
53	7	1838	56	6	2079	59	4	2341
53	8	1881	56	7	2130	59	5	2404
53	9	1924	56	8	2182	59	6	2469
53	10	1969	56	9	2235	59	7	2536
53	11	2013	56	10	2290	59	8	2604
53	12	2059	56	11	2345	59	9	2675
53	13	2105	56	12	2401	59	10	2748
53	14	2152	56	13	2459	59	11	2823
53	15	2200	56	14	2518	59	12	2900
53	16	2249	56	15	2578	59	13	2980
			56	16	2640	59	14	3063
54	0	1627	56	17	2703	59	15	3148
54	1	1668	56	18	2767	59	16	3237
54	2	1710				59	17	3328
54	3	1752	57	0	1885	59	18	3423
54	4	1794	57	1	1934	59	19	3521
54	5	1838	57	2	1984	59	20	3623
54	6	1882	57	3	2035			
54	7	1927	57	4	2087	60	0	2234
54	8	1972	57	5	2140	60	1	2296
54	9	2018	57	6	2194	60	2	2361
54	10	2065	57	7	2249	60	3	2427
54	11	2113	57	8	2305	60	4	2496
54	12	2162	57	9	2363	60	5	2567
54	13	2211	57	10	2422	60	6	2639
54	14	2261	57	11	2482	60	7	2715
54	15	2312	57	12	2544	60	8	2792
54	16	2365	57	13	2608	60	9	2873
54	17	2418	57	14	2672	60	10	2956
			57	15	2739	60	11	3042
			57	16	2807	60	12	3132
			57	17	2877	60	13	3225
			57	18	2949	60	14	3322
						60	15	3422
						60	16	3527
						60	17	3636
						60	18	3750
						60	19	3870
						60	20	3994
						60	21	4124
61	0	2383	63	0	2773			
61	1	2453	63	1	2867			
61	2	2526	63	2	2966			
61	3	2601	63	3	3071			
61	4	2678	63	4	3181			
61	5	2759	63	5	3297			
61	6	2843	63	6	3421			
61	7	2929	63	7	3552			
61	8	3020	63	8	3693			
61	9	3114	63	9	3843			
61	10	3212	63	10	4005			
61	11	3315	63	11	4179			
61	12	3422	63	12	4368			
61	13	3534	63	13	4573			
61	14	3652	63	14	4797			

Number of wells			Number of wells		
64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
61	15	3776	63	15	5039
61	16	3906	63	16	5306
61	17	4044	63	17	5598
61	18	4188	63	18	5918
61	19	4341	63	19	6267
61	20	4503	63	20	6648
61	21	4674	63	21	7063
61	22	4854	63	22	7511
			63	23	7994
62	0	2559	63	24	8513
62	1	2639	63	25	9070
62	2	2722	63	26	9666
62	3	2809	63	27	10305
62	4	2900	63	28	10991
62	5	2994	63	29	11730
62	6	3093			
62	7	3197	64	0	3045
62	8	3306	64	1	3162
62	9	3421	64	2	3286
62	10	3543	64	3	3420
62	11	3671	64	4	3564
62	12	3806	64	5	3720
62	13	3951	64	6	3889
62	14	4104	64	7	4074
62	15	4267	64	8	4277
62	16	4442	64	9	4502
62	17	4628	64	10	4753
62	18	4828	64	11	5035
62	19	5043	64	12	5352
62	20	5273	64	13	5712
62	21	5520	64	14	6119
62	22	5784	64	15	6581
62	23	6068	64	16	7101
62	24	6371	64	17	7683
62	25	6694	64	18	8329
			64	19	9043
			64	20	9826
			64	21	10687
			64	22	11636
			64	23	12687
			64	24	13864
			64	25	15199
			64	26	16740
			64	27	18563
			64	28	20795
			64	29	23671
			64	30	27726
			64	31	34659

Appendix 1 Verification of the membrane filter transfer technique for the confirmation of enterococci isolated from various waters

1 Introduction

In an earlier version of a document in this series[1] for drinking waters, the method for the confirmation of enterococci from mEA described the sub-culture of colonies onto BAA of KAAA followed by incubation at 44.0 ± 0.5°C for up to 18 hours. In addition, as some non-target organisms may also confirm as enterococci within these media and time period, the absence of catalase was also to be demonstrated.

The current ISO standard[2] for the enumeration of enterococci from water describes a confirmation procedure where the membrane filter from mEA is transferred to pre-heated (44 °C) Petri dishes containing bile aesculin azide agar (BAAA) and incubated at 44.0 ± 0.5 °C for two hours. As non-target organisms typically do not hydrolyse aesculin within this time period, testing for catalase is not included as a confirmatory requirement. In view of this, the Standing Committee of Analysts organised a study to evaluate the ISO standard approach as an alternative procedure for the confirmation of enterococci. In the study, BAA and KAAA media were used and tested with various water types in place of the BAAA medium cited in the ISO standard. The following sections describe the results of this study which was designed to demonstrate whether the two confirmation procedures are equivalent, and to optimise the membrane filter transfer procedure via assessment of the incubation time. The evaluation was undertaken in two phases. Phase 1 was a preliminary study to assess whether the two confirmation procedures gave similar results. Phase 2 of the study evaluated the comparability of the two procedures and the optimum incubation time for the membrane filter transfer technique. Details of the methodology used in the study are also included to enable laboratories to undertake their own testing.

2 Phase 1 Study

2.1 *Methodology*

Paired (i.e. duplicate) samples from surface water, crude or secondary wastewater effluents or animal slurries were analysed by membrane filtration and incubated (for most samples) on mEA at 37.0 ± 1.0°C for 4 hours followed by incubation at 44.0 ± 0.5°C for 44 ± 4 hours. For some freshwater samples, an incubation period of 37.0 ± 1.0°C for 44 ± 4 hours was used. Where required, samples were appropriately diluted and filtered to give between 10 - 30 colonies on each membrane filter. Colonies were counted in accordance with recognised MoDW procedures[1] and presumptive counts recorded.

All of the colonies from the first membrane filter incubated on mEA were selected and sub-cultured to Petri dishes containing BAA or KAAA. Petri dishes were incubated at 44.0 ± 0.5°C for up to 18 hours and the presence of aesculin hydrolysis recorded. In addition, after incubation, the absence of the enzyme catalase was demonstrated. The proportion of catalase-negative and aesculin-positive colonies was used to calculate the confirmed count. The second membrane filter from the paired (duplicate) sample was transferred from mEA to a second Petri dish of the same confirmation medium used with the first membrane filter and incubated at 44.0 ± 0.5°C for 2 hours ±

5 minutes. All colonies showing zones of aesculin hydrolysis were counted as confirmed enterococci. After counting, all colonies from transferred membrane filters were sub-cultured to fresh Petri dishes containing BAA or KAAA and incubated at 44.0 ± 0.5°C for up to 18 hours to confirm aesculin hydrolysis and the absence of catalase. Ten percent of all confirmed colonies were subjected to Gram staining to confirm that they were Gram-positive cocci. Each participating laboratory analysed 30 samples, each of which was taken through the described procedure.

2.2 *Results*

Fifteen laboratories were invited to take part in the study and twelve laboratories reported data. A total of 5611 colonies were sub-cultured for confirmation by MoDW procedures[(1)]. Of these, 5140 (i.e. 91.4 %) confirmed as enterococci. A total of 5789 colonies were transferred on membrane filters for confirmation by the modified-ISO procedure. Of these, 5042 (i.e. 86.7 %) confirmed as enterococci and 5161 (i.e. 89.2 %) confirmed by subsequent culture[(1)]. The results are summarised by sample type in Table 1.

Table 1 Rates of confirmation of presumptive enterococci by sample type

Sample type	MoDW procedures			Modified-ISO procedure				
	Presumptive count	Confirmed count	%	Presumptive count	Confirmed count	%	Subsequent confirmation by MoDW procedures	%
Primary sewage	1110	1075	96.8	1118	1007	90.1	1047	93.6
Secondary sewage	465	412	88.6	455	331	72.7	390	85.7
Surface freshwater	3276	2953	90.1	3317	2891	87.2	2925	88.2
Saline water	45	38	84.4	45	40	88.9	36	80.0
Pig slurry	305	302	99.0	297	293	98.7	297	100
Cattle faeces	61	61	100	69	69	100	69	100
Total	5262	4841	92.0	5301	4631	87.4	4764	89.9

The data from the first phase of this study suggested that the two confirmation procedures were roughly equivalent. However, the increase in the number of isolates from the modified-ISO procedure confirming as enterococci by the MoDW procedure indicated that

(a) the 2 hour incubation time may not be sufficient for the confirmation of some strains of enterococci, and

(b) that an increase in the number of aesculin-positive colonies on transferred membrane filters might be observed if the incubation period was extended to 4 or 6 hours.

3 Phase 2 Study

3.1 *Methodology*

This phase of the study involved filtering a single sample and incubating the membrane filter at 37.0 ± 1.0°C for 4 hours ± 5 minutes followed by incubation at 44.0 ± 0.5°C for 40 ± 4 hours. Alternatively, an incubation period of 37.0 ± 1.0°C for 44 ± 4

hours was used. Following incubation, a membrane filter exhibiting between 10 - 30 colonies was selected for confirmation by membrane filter transfer onto either BAA or KAAA and incubation at 44.0 ± 0.5°C for 6 hours ± 5 minutes. Colonies showing zones of aesculin hydrolysis were counted at 2 hours ± 5 minutes, 4 hours ± 5 minutes and 6 hours ± 5 minutes. All colonies showing zones of aesculin hydrolysis were counted as confirmed enterococci. At the end of the six hour incubation period, all colonies were sub-cultured to fresh Petri dishes containing BAA or KAAA and incubated at 44.0 ± 0.5°C for up to 18 hours to confirm aesculin hydrolysis, and tested for the absence of catalase.

The water types examined ranged from tap waters spiked with crude sewage (30 samples); freshwaters, for example river, stream and canal waters (133 samples); sewage effluents (107 samples); and saline waters, i.e. marine and estuarine waters (53 samples).

3.2 *Results*

The confirmation rates according to the membrane filter transfer technique with respect to water type and incubation time are summarised in Table 2. The confirmation rates at each incubation time were compared using paired data and the mean relative difference procedure with a value of D set at ten[3]. A total of 5684 colonies were subjected to testing.

It would appear that the confirmation rates for sewage effluents are lower than the confirmation rates for freshwaters and spiked tap waters, and for saline waters. This may reflect a greater range of potentially interfering false-presumptive isolates or a larger contribution to the enterococci population of species of *Enterococcus* that fail to respond on confirmation testing. Two laboratories also noted that for six samples that had large numbers of very small presumptive enterococci, the confirmation rates by membrane filter transfer were very low, even after being incubated for six hours. The majority of these isolates were confirmed as enterococci. This may reflect a slow response to the aesculin hydrolysis of some environmental strains of enterococci.

Depending on the water type, there is some difference in the confirmation rates at different incubation times. For the freshwater and spiked tap water samples, whilst the numbers of confirming colonies after 2, 4 and 6 hours of incubation increased with increasing incubation times, these were deemed “indifferent”[3]. Results that are deemed “indifferent” are those results that may give a statistically significant difference between methods, but as the confidence level in this study is within zero ± D, where D = 10, the average relative difference is too small to be of practical significance microbiologically. For sewage effluents and saline waters, the confirmation rates at 4 and 6 hours were significantly higher than the confirmation rates at 2 hours, but were “indifferent” between each other. It may be concluded, therefore, that the most appropriate incubation period for transferred membrane filters on either BAA or KAAA at 44 °C is 4 hours. An incubation period of 2 hours may be acceptable for some freshwaters and tap waters (provided they are relatively unpolluted).

Table 2 Comparison of confirmation rates of presumptive enterococci by membrane filter transfer and incubation at 44˚C

	Freshwater and spiked tap water	Sewage effluent	Saline waters	All
Number of samples	163	107	53	323
Number of presumptive counts	2954	1842	888	5684
Number confirmed at 2 hours	2632 (89.1 %)	1266 (68.7 %)	754 (84.9 %)	4717 (83.0 %)
Number confirmed at 4 hours	2769 (93.7 %)	1474 (80.0 %)	826 (93.0 %)	5137 (90.4 %)
Number confirmed at 6 hours	2799 (94.8 %)	1542 (83.7 %)	834 (93.9 %)	5243 (92.2 %)
4 hours versus 2 hours				
Mean relative difference (%)	5.66	19.69	10.50	10.85
χ lower	3.95	13.55	5.85	8.50
χ higher	7.37	25.83	15.14	13.21
Outcome	Indifferent*	4-hour results significantly higher	4-hour results significantly higher	4-hour results significantly higher
6 hours versus 2 hours				
Mean relative difference (%)	7.25	24.84	11.25	13.40
χ lower	5.39	17.30	6.60	10.60
χ higher	9.11	32.38	15.90	16.21
Outcome	Indifferent*	6-hour results significantly higher	6-hour results significantly higher	6-hour results significantly higher
6 hours versus 4 hours				
Mean relative difference (%)	1.59	5.14	0.75	2.55
χ lower	0.87	2.99	0.12	1.75
χ higher	2.30	7.29	1.39	3.35
Outcome	Indifferent*	Indifferent*	Indifferent*	Indifferent*

* Indifferent - Although the shorter incubation period gave a statistically significant lower number of confirming isolates, the average relative difference is probably too small to be microbiologically significant at a practical level.

The data for confirmation by membrane filter transfer with incubation at 44 °C for 4 or 6 hours were compared to those for the isolates subsequently sub-cultured for confirmation by MoDW procedures[(1)]. These data are summarised in Table 3.

For freshwaters and spiked tap waters, and saline waters, confirmation by membrane filter transfer at 44°C for 4 or 6 hours is not significantly different from confirmation by MoDW procedures[(1)]. For sewage effluents, confirmation by MoDW procedures[(1)] was significantly higher than confirmation by membrane filter transfer at 44°C for 4 hours, but, although still higher, was “indifferent” compared to confirmation by membrane filter transfer at 44°C for 6 hours. When all data is combined, the overall conclusion is that confirmation by the membrane filter transfer technique at 44°C for 4 hours was “indifferent” to confirmation by MoDW procedures. However, for confirmation by the membrane filter transfer technique at 44°C for 6 hours there was no statistical significant difference to confirmation by MoDW procedures. These data do show however that the matrix of the sample may play a significant role in the performance of the confirmation procedure. Overall, the membrane filter transfer technique with

incubation at 44°C for 4 hours can be considered equivalent to the MoDW procedures[1] for some waters, but incubation at 44°C for 6 hours may be more appropriate for sewage effluents.

Table 3 Comparison of confirmation rates of presumptive enterococci by membrane filter transfer (MFT) and incubation at 44˚C for 4 or 6 hours and by MoDW procedures[1]

	Freshwaters and spiked tap waters	Sewage effluent	Saline waters	All
Number of samples	163	107	53	323
Number of presumptive counts	2954	1842	888	5684
Number confirmed by MoDW	2757 (93.3 %)	1696 (92.1 %)	834 (93.9 %)	5287 (93.0 %)
Number confirmed by MFT at 4 hours	2769 (93.7 %)	1474 (80.0 %)	826 (93.0 %)	5137 (90.4 %)
Number confirmed by MFT at 6 hours	2799 (94.8 %)	1542 (83.7 %)	834 (93.9 %)	5243 (92.2 %)
MFT at 4 hours versus MoDW				
Mean relative difference (%)	- 0.99	- 10.32	- 1.12	- 4.10
χ lower	- 3.60	-13.52	- 4.28	- 5.99
χ higher	1.61	- 7.12	0.73	- 2.22
Outcome	no statistical significant difference	MoDW results significantly higher	no statistical significant difference	Indifferent*
MFT at 6 hours versus MoDW				
Mean relative difference (%)	0.59	- 5.42	- 0.37	- 1.56
χ lower	- 1.69	- 7.73	- 4.56	- 3.12
χ higher	2.87	- 3.11	3.82	0.01
Outcome	no statistical significant difference	Indifferent*	no statistical significant difference	no statistical significant difference

* Indifferent - Although the results by the MoDW procedures gave a statistically significant higher number of confirming isolates, the average relative difference is probably too small to be microbiologically significant at a practical level.

4 Conclusions

For some waters, particularly the freshwaters, tap waters and saline waters tested, a modified-ISO standard membrane filter transfer technique using BAA or KAAA has been shown to be an equivalent alternative procedure to those previously described in this series[1] for the confirmation of enterococci. An incubation period of 4 hours $\pm$ 5 minutes has been shown to be more appropriate than the 2 hours cited in the ISO standard[2]. For sewage samples, it may be that 6 hours $\pm$ 5 minutes is more appropriate to demonstrate the equivalency of the MoDW procedures[1] with the membrane filter transfer technique using BAA or KAAA.

5 Quality Control

All of the media used in the study was prepared and checked in accordance with procedures described elsewhere[4]. A known strain of *Enterococcus faecalis* (for example, NCTC 775) was included as a positive control for the whole procedure.

6 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2010) - Part 5 - Isolation and enumeration of Enterococci by membrane filtration, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

2. BS EN ISO 7899-2:2000 - Water quality - Detection and enumeration of intestinal enterococci - Part 2 - Membrane filtration method. Geneva: International Organisation for Standardisation.

3. ISO 17994:2004 Water quality – Criteria for establishing equivalence between microbiological methods. Geneva: International Organisation for Standardisation.

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*, Environment Agency.

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Address for correspondence

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Environment Agency (National Laboratory Service)
NLS Nottingham
Meadow Lane
Nottingham
NG2 3HN
(http://www.gov.uk/environment-agency)

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