ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2012) - Part 5 –Methods for the Isolation and enumeration of enterococci

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 two methods for the isolation and enumeration of enterococci and replaces the earlier booklet published in 2010.

A The enumeration of enterococci by membrane filtration.

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

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as an illustrative example of the type of products available. Equivalent products are 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.

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

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 3 - Practices and procedures for laboratories (currently undergoing revision)
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

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

The Microbiology of Drinking Water (2007)
Part 7 - Methods for the enumeration of heterotrophic bacteria (currently undergoing revision)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2009)
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
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 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

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.

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.

In the procedures described in each method any reference to the tolerances to be adopted with respect to, for example the amount or volume of reagents to be used is left to the discretion of the laboratory. These tolerances should be as low as possible in order to satisfy stringent performance criteria. Tolerances of between 1 - 5 % have been shown to be satisfactory for most purposes. Lower tolerances should result in improved precision.

In the methods described, for example for wavelengths, storage conditions, concentrations of the same or similar reagents, etc, differences may be noted. This information is provided by individual laboratories operating under their own management systems and is dependent on specific conditions pertaining to each laboratory. It is assumed this information is supported by sufficient data to justify its inclusion. Users intending to use or vary the quoted wavelengths, storage conditions, concentrations, etc, should ensure they are appropriate to their own laboratory and verify their application to demonstrate appropriate performance of the method. 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.

Methods are produced by panels of experts in the appropriate field, often in co-operation with working groups and the main committee. The names of those members principally associated with these methods are listed at the back of this booklet. A report describing all SCA activities for the period 1 July to 30 June is produced annually and is available from the Agency’s web-page (www.environment-agency.gov.uk/nls).

Users should ensure they are aware of the most recent version of the draft they seek. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency’s internet web-page or by post, see address listed at the back of this booklet.

Great efforts are made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Mark Gale
Secretary
March 2012

Warning to users

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

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

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

A The enumeration of enterococci by membrane filtration

A1 Introduction

In the United Kingdom, enterococci are regarded as secondary indicators of faecal pollution, and the main use of the test for enterococci is to assess the significance of coliform bacteria in a sample in the absence of *Escherichia coli* (*E. coli*). Occasionally, identification of the species of enterococci or streptococci in a sample may help to distinguish between human and animal pollution. The significance of enterococci in water treatment and supply are described elsewhere[(1)] in this series.

A2 Scope

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

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

A3 Definitions

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 (mEA)[(3)] when incubated at 37 °C or 44 °C. Some strains may produce colonies which are very small and/or pale in colour. Confirmation is based[(4)] on the demonstration of aesculin hydrolysis on bile aesculin agar (BAA) or kanamycin aesculin azide agar (KAAA) by sub-culturing colonies from mEA to BAA or KAAA and incubating at 44 °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 °C for 4 hours. Some strains of enterococci and some strains of *Streptococcus bovis* and *Streptococcus equinus*, whilst growing at 37 °C on mEA may fail to grow at 44 °C.

Enterococci are Gram-positive cocci which form pairs or chains, possess Lancefield's Group D antigen and are catalase-negative. The organisms grow in the presence of bile salts, in concentrations of sodium azide that are inhibitory to coliform bacteria and most other Gram-negative bacteria, and at a temperature of 44 °C. They also express the enzyme β-glucosidase. Some species display other characteristics useful for identification, such as resistance 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.

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

growth of organisms. Other bacteria (for example, *Aerococcus viridans* and species of *Staphylococcus* and *Bacillus*) are also able to grow under the conditions described. The maximum number of typical and non-typical colonies that should be present on a single membrane filter from which counts are estimated should be approximately 200[5]. 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 in excess of 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 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, containing a sufficient quantity of a suitable de-chlorinating agent, for example a solution of sodium thiosulphate pentahydrate. This can be used 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).

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, filter funnels (either sterilised or capable of being sterilised, and vacuum source).

A7.4 Sterile, membrane filters, for example, white, 47 mm diameter, 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 quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of media is not within the stated range, then, before heating, they should be adjusted accordingly.

A8.1 *Membrane enterococcus agar*

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 one month, if protected against dehydration.

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

A8.2 *Bile aesculin agar*

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 pH value of the solution to 7.1 ± 0.2. Sterilise the solution at 121 °C for 15 minutes. The final pH of the cooled solution should be 7.1 ± 0.2. Sterile media may be stored for up to one month at a temperature of 5 ± 3 °C, if protected against dehydration.

A8.3 *Kanamycin aesculin azide agar*

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 pH of the cooled solution should be 7.0 ± 0.2. Sterile media may be stored for up to one month at a temperature of 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, quarter strength Ringer's solution and maximum recovery diluent.

A9 Analytical procedure

A9.1 *Sample preparation*

For treated waters, filter 100 ml of the sample. For polluted waters, filter smaller volumes, or dilute the sample with maximum recovery diluent or quarter strength Ringer's solution before filtration. 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 so that the number of colonies on any single membrane filter lies within this range.

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, for example 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 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 been 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.

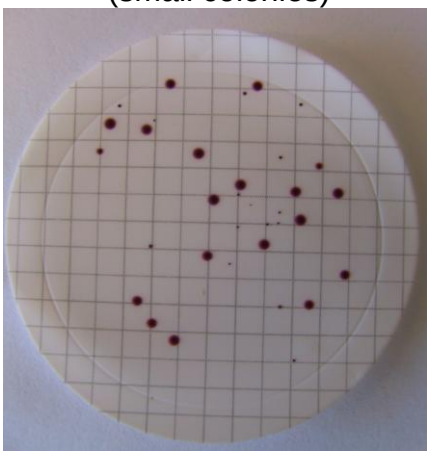
Encouraging the growth of enterococci is a balance between selectivity and sensitivity. The growth of enterococci on mEA is better at 37 °C, although some organisms resembling enterococci may also grow on this medium at this temperature. Selectivity is better at 44 °C although lower counts of enterococci may be obtained, as some strains do not grow or do not grow well at 44 °C. It may be more appropriate if membrane filters from samples of potable water are incubated at 37.0 ± 1.0 °C for 44 ± 4 hours, whilst membrane filters from samples of untreated water are incubated at 37.0 ± 1.0 °C for 4.0 ± 0.5 hours followed by 44.0 ± 0.5 °C for 40 ± 4 hours. Petri-dishes should be incubated inverted at the selected incubation temperature.

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 1). These colonies are regarded as presumptive enterococci. Some types of enterococci may produce very pale colonies. Colonial size is variable. Some species of *Bacillus* may produce pink colonies but these are often rough, flat and sometimes spread over the surface of the agar. Some species of *Aerococcus* and *Staphylococcus* can also grow on mEA producing red colonies.

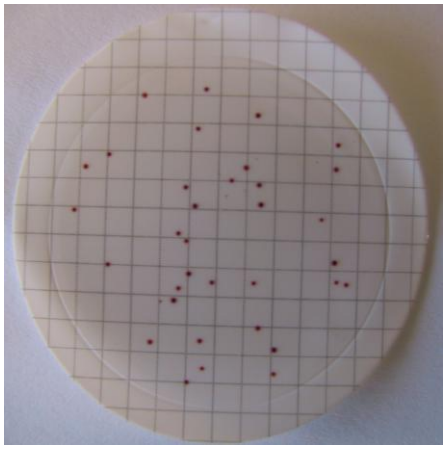
Figure 1 Colonies on membrane enterococcus agar

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

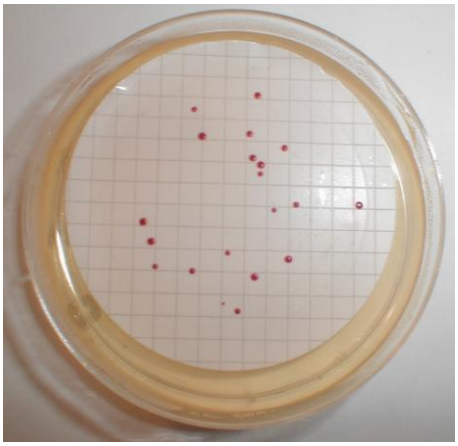


Colonial size difference is not always apparent.

Enterococcus casseliflavus



Enterococcus faecalis



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 4 hours ± 5 minutes. See flow chart.

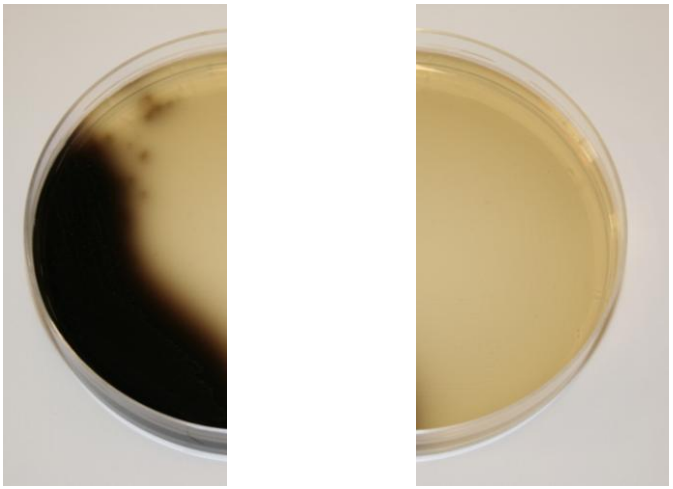
A9.4.1 Aesculin hydrolysis by sub-culture

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, subculture 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 resulting from aesculin hydrolysis. See Figure 2. 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.

Figure 2 Colonies on kanamycin aesculin azide agar

Enterococcus faecalis *Aerococcus viridans*



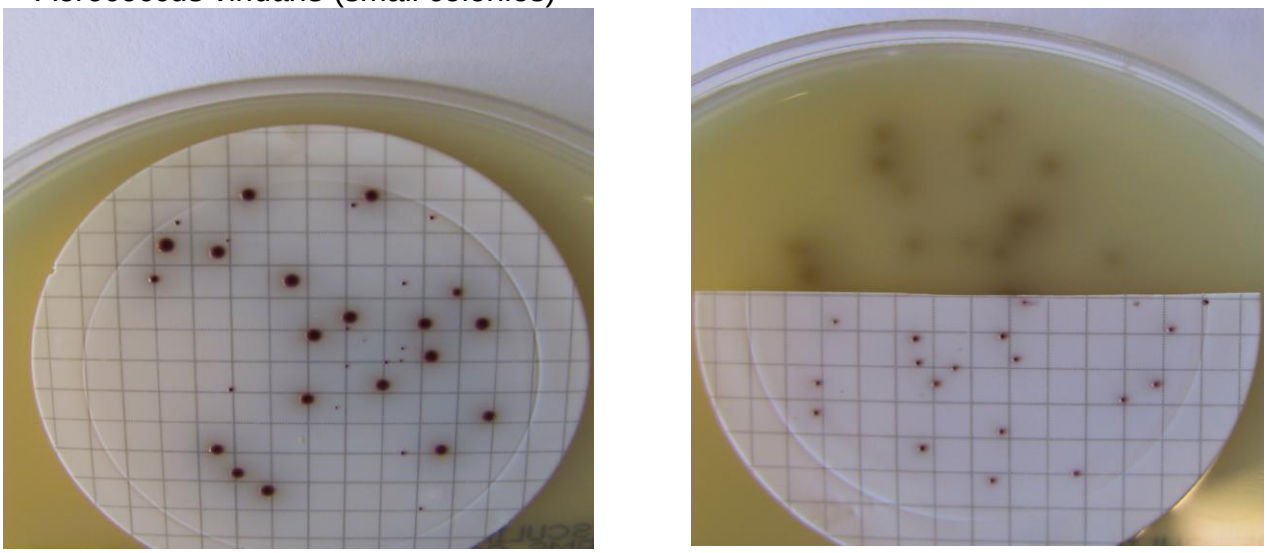
A9.4.2 Aesculin hydrolysis by membrane transfer

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 4 hours ± 5 minutes. Data on the verification of the performance of this confirmation procedure are given in appendix 1. 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 3). The development of this halo is often evident within 2 hours and may provide rapid confirmation, but see appendix 1. However, some strains may take longer to produce the coloration, and hence the need for further incubation.

Figure 3 Colonies on bile aesculin agar

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

Enterococcus casseliflavus



Colonial size difference is not always apparent.

A9.5 *Additional differentiation tests for enterococci*

If additional tests are to be conducted, and depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of aesculin-positive colonies. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured (if fewer than ten 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. Further differentiation is not usually necessary for routine water testing, but may be appropriate where particular problems are encountered. 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 can also be 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, and their resistance to heating at 60 °C.

A9.5.1 *Catalase test*

Enterococci are catalase-negative. For each sub-culture to be tested, from the sub-cultured BAA or KAAA Petri dish (9.4.1) or the membrane filter incubated on BAA or KAAA (9.4.2) into a small screw-capped bottle, emulsify some of the isolated colony in approximately 0.1 ml of quarter strength Ringer's solution. 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 sub-culture from mEA to nutrient agar, brain heart infusion agar, Mueller Hinton agar or similar non-selective medium and incubate overnight at 37 °C. To a pure culture obtained from the nutrient agar, brain heart infusion agar, Mueller Hinton agar or similar non-selective medium add approximately 0.05 ml of 5 - 6 v/v % hydrogen peroxide solution. The immediate appearance of bubbles (of oxygen) indicates catalase activity. Isolates should not be taken from 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.

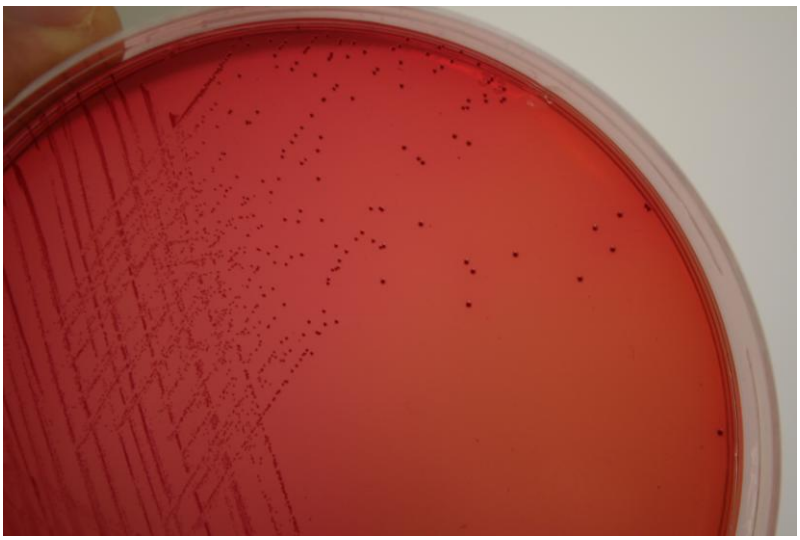
On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *E. coli*,

Pseudomonas aeruginosa or *Staphylococcus aureus*) and one species is known to give a negative reaction (for example, *Enterococcus faecalis*).

A9.5.2 *Bile tolerance*

From an overnight culture (previously isolated from BAA or KAAA) on nutrient agar, incubated at 37 °C, 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 4). 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 4 Colonies of *Enterococcus faecalis* on MacConkey agar



A9.5.3 *Heat resistance*

From BAA or KAAA, 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 is known to survive this heat treatment (for example, *Enterococcus faecalis*) and one species is known not to survive (for example, *Streptococcus bovis* or *Streptococcus equinus*).

A9.5.4 *Growth at pH 9.6*

From BAA or KAAA, transfer appropriate colonies to a Petri dish containing nutrient agar and incubate overnight at 37 °C. Transfer a colony from the nutrient agar and inoculate into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 37 °C for 21 ± 3 hours. Tolerance to this solution (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*

From BAA or KAAA, transfer appropriate colonies to a Petri dish containing nutrient agar and incubate overnight at 37 °C. 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 is known not to grow in the presence of 6.5 % salt (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:

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

Where DF is the dilution factor, if appropriate.

A10.2 *Confirmed enterococci*

The number of confirmed enterococci is the number of presumptive enterococci on mEA which on transfer to BAA or KAAA hydrolyse aesculin.

A11 Expression of results

Presumptive and confirmed enterococci are expressed in colony forming units per volume of sample. For drinking water the volume is typically 100 ml.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Enterococcus faecalis*) and non-target bacteria (for example, *Staphylococcus warneri* strain WR51. This strain will grow on membrane enterococcus agar if the sodium azide concentration is less than 300 mg/l). Petri dishes should be incubated at 37 °C for 44 ± 4 hours. Further details are given elsewhere[2] in this series.

A13 References

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

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency. (currently undergoing revision.)

3. Numbers of enterococci in water, sewage and faeces determined by the membrane filter technique with an improved medium. L W Slanetz and C H Bartley, *Journal of Bacteriology*, 1957, **74**, pp591-595.

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

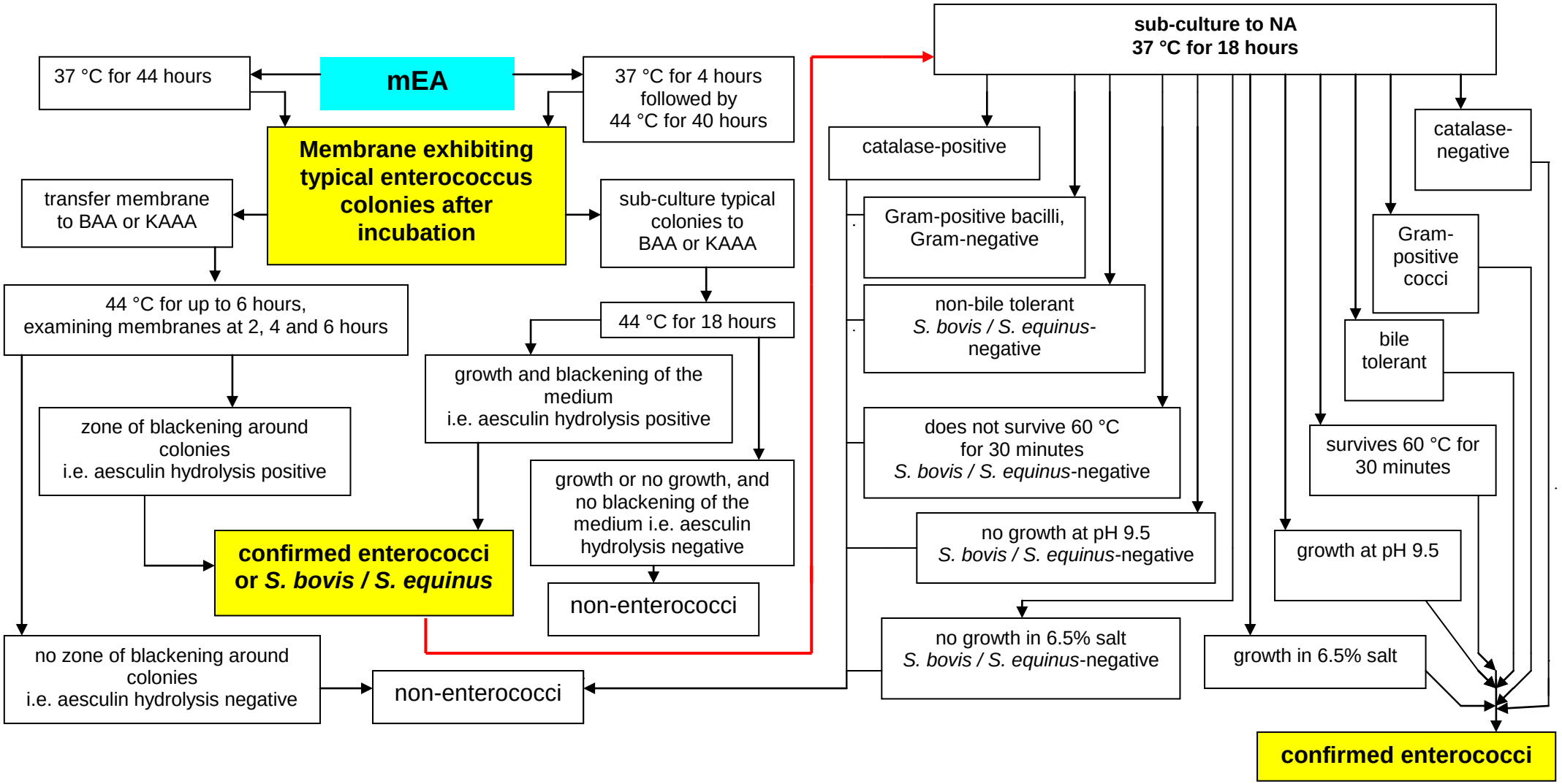
5. ISO 8199:2005 Water quality – General guidance on the enumeration of micro-organisms by culture. Geneva: International Organisation for Standardisation

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, P H Clarke, *Journal of General Microbiology*, 1953, **9**, pp350-352.

Flow chart **Confirmation of presumptive enterococcus colonies isolated on membrane enterococcus agar when incubated at 37 °C for 44 hours or 37 °C for 4 hours followed by 44 °C for 40 hours** (see sections A9.4 and A9.5)



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

B1 Introduction

In the United Kingdom, enterococci are regarded as secondary indicators of faecal pollution, and the main use of the test for enterococci is to assess the significance of coliform bacteria in a sample in the absence of *Escherichia coli* (*E. coli*). Occasionally, identification of the species of enterococci in a sample may help to distinguish between human and animal pollution. The significance of enterococci in water treatment and supply 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 drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate to high turbidity.

Users wishing to employ this method should verify its 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[3], as demonstrated by a colour change to green, through the enzymatic cleavage of ortho-nitrophenyl-β-D-glucoside (by the production of a yellow compound in a broth originally having a blue colour) in a defined substrate medium, are regarded as enterococci.

Enterococci are Gram-positive cocci which form pairs or chains, possess Lancefield's Group D antigen and are catalase-negative. The organisms grow in the presence of bile salts, in concentrations of sodium azide that are inhibitory to coliform bacteria and most other Gram-negative bacteria, and at a temperature of 44 °C. They also express the enzyme β-glucosidase. Some species display other characteristics useful for identification, such as resistance 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

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 dilution of sample, which is then added to a 51-well reaction pouch. This is then sealed and incubated at 41 °C for 24 hours. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth as indicated by a colour change, then the most probable number of enterococci in 100 ml of sample can be estimated from appropriate probability tables, see Table B1.

B5 Limitations

This method is suitable for most types of water samples. Those with high turbidities, however, may mask or impede colour development. Waters with a high mineral content may result in the sample appearing cloudy but this does not affect the reliability of the test nor the ability to read positive reactions.

B6 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[2] in this series.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series. Principally, a fan assisted incubator is 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 bottles of appropriate volume, made of suitable material, containing a sufficient quantity of a suitable de-chlorinating agent, for example a solution of sodium thiosulphate pentahydrate. This can be used 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).

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) and associated heat-sealing equipment.

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

B8.1 *Enterolert®-DW medium*[5].

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 is used in conjunction with the 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 treated 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 only sterile, non-buffered, oxidant-free water. Buffered solutions should not be used as they may adversely affect the performance of the medium.

B9.2 *Sample processing*

The sample, or appropriate dilution, (usually 100 ml) is decanted into a sterile bottle containing anti-foaming 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, typically, for a few minutes to allow completion of dissolution and dispersal of any air bubbles. 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. Prolonged exposure of the inoculated reaction pouch 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 ± 0.5 °C for not less than 24 hours and not more than 28 hours.

B9.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that have changed to a green colour from the original blue colour (see Figure 1) are recorded as positive reactions. If the pouch is examined and 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.

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 method A).

Figure 1 Example of a 51-well MPN reaction pouches (Quanti-Tray®) with a defined substrate medium (Enterolert®-DW) with 18 positive green wells for enterococci

B10 Calculations

B10.1 *Confirmed enterococci*

The MPN of enterococci is determined by reference to appropriate probability tables, see for example Table B1. This is derived from the number of wells showing a positive, green colouration. For example, if there are 18 wells showing a green coloration in the reaction pouch (as shown in Figure 1), then from Table B1, the MPN of enterococci is 22 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. For drinking water, 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 faecium* NCTC 7176/ATCC 35667) and non-target bacteria (for example, *Serratia marcescens* ATCC 43862). Pouches should be incubated for 24-28 hours at 41 ± 0.5 °C. Further details are given elsewhere[(2)] in this series.

B13 References

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

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

3. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. K J Domig, H K Mayer & W Kneifel, 2003, **88**, 165-188.

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

5. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9EW.

Table 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	

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

1 Introduction

In an earlier edition of this document[1], the method for the confirmation of enterococci from mEA described the subculture 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 subcultured 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	98.6	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
	5611	5140	91.4	5789	5042	86.7	5161	89.2

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.

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 (71.9 %)	754 (84.9 %)	4717 (83.0 %)
Number confirmed at 4 hours	2769 (93.7 %)	1474 (83.7 %)	826 (93.0 %)	5137 (90.4 %)
Number confirmed at 6 hours	2799 (94.8 %)	1542 (87.6 %)	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 give a statistically significant lower number of confirming isolates, the average relative difference is probably too small to be microbiologically significant at a practical level.

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 $\pm$ 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).

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.

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 (83.7 %)	826 (93.0 %)	5137 (90.4 %)
Number confirmed by MFT at 6 hours	2799 (94.8 %)	1542 (87.6 %)	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.

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.

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 ± 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 ± 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 (2002) - Part 5 - Isolation and enumeration of Enterococci by membrane filtration, *Methods for the Examination of Waters and Associated Materials*, in this series, 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*, in this series, Environment Agency.

7 Acknowledgements

The Standing Committee of Analysts is indebted to the managers and analysts of the following laboratories that participated in this study:

CREH *Analytical* (Leeds)
Environment Agency (National Laboratory Service, Exeter)
Northern Ireland Water Services (Londonderry)
Severn Trent Laboratories (Bridgend)
Severn Trent Water (Nottingham)
Severn Trent Water (Shrewsbury)
South West Water (Exeter)
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United Utilities (Warrington)
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P Boyd	Health Protection Agency
S Cole	Wessex Water
D Gaskell	United Utilities Scientific Services
D Sartory	SWM Consulting
H.Shapland	Wessex Water
J Watkins	CREH *Analytical* Ltd

Grateful acknowledgement is made to P Boyd, J Watkins and IDEXX Laboratories for providing colour photographs.