





## **The Microbiology of Drinking Water (2006) - Part 5 - The isolation and enumeration of enterococci by membrane filtration**

Methods for the Examination of Waters and Associated Materials

This booklet contains a method for the isolation and enumeration of enterococci by membrane filtration and describes an alternative and equivalent confirmation procedure to that previously described in The Microbiology of Drinking Water (2002) - Part 5 - The isolation and enumeration of enterococci by membrane filtration.

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

The Microbiology of Drinking Water (2002)

Part 1 - Water quality and public health

Part 2 - Practices and procedures for sampling

Part 3 - Practices and procedures for laboratories

Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)

Part 5 - The isolation and enumeration of enterococci by membrane filtration

Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques

Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration

Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)

Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration

Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques

Part 11 - Taste, odour and related aesthetic problems

Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

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.

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## About this series

### Introduction

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

### Performance of methods

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

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

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

### Standing Committee of Analysts

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

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

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

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this method 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 advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page ([www.environment-agency.gov.uk/nls](http://www.environment-agency.gov.uk/nls)) or by post.

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

Dr D Westwood  
*Secretary*  
January 2006

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## Warning to users

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

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

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

## The isolation and enumeration of enterococci by membrane filtration

### 1 Introduction

In the United Kingdom, enterococci are regarded as a second primary indicator 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<sup>(1)</sup> in this series.

### 2 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<sup>(2)</sup>.

### 3 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<sup>(3)</sup> 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 on procedures previously described<sup>(4)</sup> or alternatively, on the demonstration of aesculin hydrolysis on bile aesculin agar (BAA) or kanamycin aesculin azide agar (KAAA) by transferring the membrane filter<sup>(5)</sup> from membrane enterococcus agar (mEA) 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 organism grows 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. 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.

### 4 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.

### 5 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, *Acrococcus 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<sup>(6)</sup>. 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.

## **6 Health and safety**

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(7)</sup> and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(2)</sup> 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.

## **7 Apparatus**

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

7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) per 100 ml of sample, or equivalent may be suitable.

7.2 Incubators, capable of maintaining temperatures of  $37.0 \pm 1.0$  °C and  $44.0 \pm 0.5$  °C or cycling incubators, fitted with timers, capable of attaining these temperatures.

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

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

7.5 Smooth-tipped forceps.

## **8 Media and reagents**

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should



be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly.

#### 8.1 *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  $7.2 \pm 0.2$ . Filter-sterilise the TTC solution through a nominal  $0.2 \mu\text{m}$  membrane filter. Cool the medium to  $50 \text{ }^\circ\text{C}$  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  $5 \pm 3 \text{ }^\circ\text{C}$  for up to one month, if protected against dehydration.

Care should be taken to avoid overheating the medium, as this may result in a deterioration of its performance. This is due to the breakdown of TTC and sodium azide. If the medium is pink when cooled to  $50 \text{ }^\circ\text{C}$ , then it should be discarded.

#### 8.2 *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  $7.1 \pm 0.2$ . Sterilise the solution at  $121 \text{ }^\circ\text{C}$  for 15 minutes. The final pH of the cooled solution should be  $7.1 \pm 0.2$ . Sterile media may be stored for up to one month at a temperature of  $5 \pm 3 \text{ }^\circ\text{C}$ , if protected against dehydration.



### 8.3 *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  $7.0 \pm 0.2$ . Sterile media may be stored for up to one month at a temperature of  $5 \pm 3$  °C, if protected against dehydration.

### 8.4 *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, Ringer's solution and maximum recovery diluent.

## 9 Analytical procedure

### 9.1 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.

### 9.2 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 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.

## 9.5 Additional differentiation tests for enterococci

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<sup>(8)</sup>. 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<sup>(9)</sup> modified to pH 9.6.

### 9.5.1 Catalase test

Enterococci are catalase-negative. For each colony to be tested from the membrane filter incubated on BAA or KAAA, 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 3 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 the hydrogen peroxide to an overnight culture (incubated at 37 °C) of an isolate obtained from nutrient agar, brain heart infusion agar, Mueller Hinton agar or similar non-selective medium. 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.

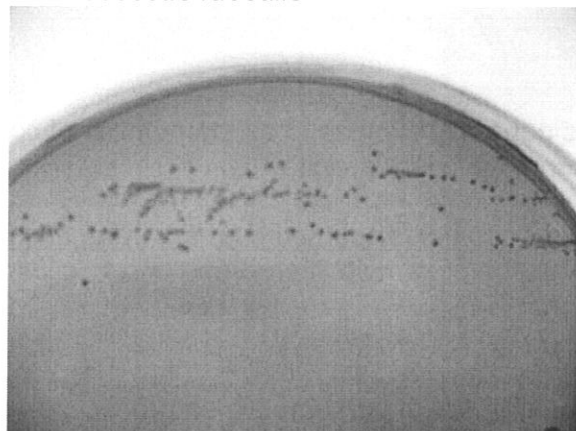
On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Staphylococcus aureus*) and one species is known to give a negative reaction (for example, *Enterococcus faecalis*).

### 9.5.2 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 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 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 3). 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 3** Colonies on MacConkey agar

*Enterococcus faecalis*



#### 9.5.3 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. Subculture 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*).

#### 9.5.4 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 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*).

#### 9.5.5 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*).

## 10 Calculations

### 10.1 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:

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

### 10.2 Confirmed enterococci

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

## 11 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.

## 12 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<sup>(2)</sup> in this series.

## 13 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. 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. 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.
5. BS EN ISO 7899-2:2000 Water quality – Detection and enumeration of intestinal enterococci – Part 2 – Membrane filtration method. Geneva: International Organisation for Standardisation.

6. ISO 8199:2005 Water quality – General guidance on the enumeration of micro-organisms by culture. Geneva: International Organisation for Standardisation
7. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.
8. *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.
9. Growth of streptococci in a glucose phenolphthalein broth, P H Clarke, *Journal of General Microbiology*, 1953, **9**, pp350-352.

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

### **1 Introduction**

In a previous document<sup>(1)</sup> in this series procedures are described for the two-stage confirmation of enterococci from mEA by subculture of colonies to BAA or KAAA and incubation at  $44.0 \pm 0.5$  °C for up to 18 hours. A catalase test is then carried out to distinguish enterococci from non-target organisms that may hydrolyse aesculin within these media and time period.

The current ISO standard<sup>(2)</sup> for the isolation and enumeration of enterococci from water describes a confirmation procedure where the membrane filter from mEA is transferred to a pre-heated (44 °C) Petri dish containing bile aesculin azide agar (BAAA) and incubated at  $44.0 \pm 0.5$  °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, a multi-laboratory study to evaluate the ISO approach as an alternative procedure for the confirmation of enterococci was organised by the Standing Committee of Analysts. 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 study was designed to determine whether the two confirmation procedures were comparable, and if so, 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 optimum incubation time for the membrane filter transfer technique and the comparability of the two procedures. Details of the methodology used in the study are 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  $37.0 \pm 1.0$  °C for 4 hours followed by incubation at  $44.0 \pm 0.5$  °C for  $44 \pm 4$  hours. For some freshwater samples, an incubation period of  $37.0 \pm 1.0$  °C for  $44 \pm 4$  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<sup>(1)</sup> 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  $44.0 \pm 0.5$  °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  $44.0 \pm 0.5$  °C for 2 hours  $\pm$  5 minutes. All colonies showing zones of aesculin hydrolysis were counted as confirmed enterococci. After counting, all colonies from transferred membrane filters were subcultured to fresh Petri dishes containing BAA or KAAA and incubated at  $44.0 \pm 0.5$  °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 subcultured for confirmation by MoDW procedures<sup>(1)</sup>. 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<sup>(1)</sup>. The results, collated according to sample matrix, are summarised 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 comparable. 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  $37.0 \pm 1.0$  °C for 4 hours  $\pm$  5 minutes followed by incubation at  $44.0 \pm 0.5$  °C for  $40 \pm 4$  hours. Alternatively, an incubation period of  $37.0 \pm 1.0$  °C for  $44 \pm 4$  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  $44.0 \pm 0.5$  °C for 6 hours  $\pm$  5 minutes. Colonies showing zones of aesculin hydrolysis were counted at 2 hours  $\pm$  5 minutes, 4 hours  $\pm$  5 minutes and 6 hours  $\pm$  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 subcultured to fresh Petri dishes containing BAA or KAAA and incubated at  $44.0 \pm 0.5$  °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  $10^{-3}$ . 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
$\chi$ lower	3.95	13.55	5.85	8.50
$\chi$ 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
$\chi$ lower	5.39	17.30	6.60	10.60
$\chi$ 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
$\chi$ lower	0.87	2.99	0.12	1.75
$\chi$ 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

procedures<sup>(1)</sup>. For sewage effluents, confirmation by MoDW procedures<sup>(1)</sup> 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<sup>(1)</sup> 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 fresh and tap 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<sup>(1)</sup> for the confirmation of enterococci. However, 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<sup>(2)</sup>.

To demonstrate similar equivalency of the MoDW procedures<sup>(1)</sup> with the membrane filter transfer technique using BAA or KAAA for sewage samples, it may be that 6 hours  $\pm$  5 minutes is more appropriate than the 2 hours cited in the ISO standard<sup>(2)</sup>.

#### 5 Quality Control

All of the media used in the study was prepared and checked in accordance with procedures described elsewhere<sup>(4)</sup>. 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.

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"<sup>(3)</sup>. Results that are deemed "indifferent" are those results that give a statistically significant difference between methods, but as the confidence interval in this study is within zero  $\pm$  D, where D = 10, the average relative difference is considered 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 sewage effluents and saline waters 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 subcultured for confirmation by MoDW procedures<sup>(1)</sup>. 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<sup>(1)</sup>**

	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
$\chi$ lower	- 3.60	-13.52	- 4.28	- 5.99
$\chi$ 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
$\chi$ lower	- 1.69	- 7.73	- 4.56	- 3.12
$\chi$ 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

## 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)

Southern Water (Brighton)

Thames Water (Reading)

United Utilities (Warrington)

Veolia Partnership (Watford)

Wessex Water (Bath)

### **Address for correspondence**

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Secretary  
Standing Committee of Analysts  
Environment Agency (National Laboratory Service)  
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Rothley  
Leicestershire  
LE7 7NW  
[www.environment-agency.gov.uk/nls](http://www.environment-agency.gov.uk/nls)

### **Environment Agency Standing Committee of Analysts Members assisting with this method**

P Boyd  
S Cole  
D Sartory  
J Watkins  
C Weatherley

Grateful acknowledgement is made to Peter Boyd of the Health Protection Agency, Newcastle for providing coloured photographs.

## CONTACTS:

### ENVIRONMENT AGENCY HEAD OFFICE

Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol BS32 4UD

[www.environment-agency.gov.uk](http://www.environment-agency.gov.uk)

[www.environment-agency.wales.gov.uk](http://www.environment-agency.wales.gov.uk)

### ENVIRONMENT AGENCY REGIONAL OFFICES

#### ANGLIAN

Kingfisher House  
Goldhay Way  
Orton Goldhay  
Peterborough PE2 5ZR

#### MIDLANDS

Sapphire East  
550 Streetsbrook Road  
Solihull B91 1QT

#### NORTH EAST

Rivers House  
21 Park Square South  
Leeds LS1 2QG

#### NORTH WEST

PO Box 12  
Richard Fairclough House  
Knutsford Road  
Warrington WA4 1HG

#### SOUTHERN

Guildbourne House  
Chatsworth Road  
Worthing  
West Sussex BN11 1LD

#### SOUTH WEST

Manley House  
Kestrel Way  
Exeter EX2 7LQ

#### THAMES

Kings Meadow House  
Kings Meadow Road  
Reading RG1 8DQ

#### WALES

Cambria House  
29 Newport Road  
Cardiff CF24 0TP



ENVIRONMENT AGENCY  
GENERAL ENQUIRY LINE

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