# Standing Committee of Analysts

## The Microbiology of Recreational and Environmental Waters (2015) – Part 6 – Methods for the isolation and enumeration of *Staphylococcus aureus*

Methods for the Examination of Waters and Associated Materials

**The Microbiology of Recreational and Environmental Waters (2015) – Part 6 – Methods for the isolation and enumeration of *Staphylococcus aureus***

**Methods for the Examination of Waters and Associated Materials**

This booklet contains methods for the isolation and enumeration of *Staphylococcus aureus* by membrane filtration and a most probable number technique.

A Method for the isolation and enumeration of *Staphylococcus aureus* by membrane filtration

B Method for the isolation and enumeration of *Staphylococcus aureus* by a presence-absence or multiple tube most probable number technique

This bluebook updates and replaces section 7.9 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products. They serve only as illustrative examples of the types of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

**Contents**

## About this series

### Introduction

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

### Performance of methods

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

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

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

### Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are seven working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (http://standingcommitteeofanalysts.co.uk/) or by post.

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

Robert Carter
*Secretary*
June 2015

---

## Warning to users

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

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.
Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
"Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

# A Method for the isolation and enumeration of *Staphylococcus aureus* by membrane filtration

## A1 Introduction

Staphylococci are widely distributed in the environment and may be isolated from foods, faeces, and the skin and mucous membranes of warm blooded animals. The organisms are opportunistic pathogens of man causing non-diarrhoeal diseases such as eye, ear, nose and skin infections, and as a consequence their isolation and enumeration may be required, particularly from swimming pools, spa pools and hydrotherapy pools. The significance of *Staphylococcus aureus* in recreational and other waters is described elsewhere[1] in this series.

## A2 Scope

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

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 *Staphylococcus aureus* are defined as those bacteria which produce black or dark grey colonies on Vogel-Johnson agar[3] after incubation at 37°C.

Confirmed *Staphylococcus aureus* are characteristic colonies obtained from Vogel-Johnson agar which, when Gram-stained, appear as Gram-positive cocci resembling "bunches of grapes" and which are coagulase-positive.

## A4 Principle

Organisms are entrapped on a membrane filter which is then placed on the surface of an agar medium containing potassium tellurite and lithium chloride as selective agents, and mannitol as a fermentable carbohydrate. *Staphylococcus aureus* produces black or dark grey colonies as a result of the reduction of the tellurite to metallic tellurium.

## A5 Limitations

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. In such instances, the sample volume can be increased by the use of several membrane filters or filter-aid used. When low numbers of organisms are present, detection is dependent only on the volume of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms.

Accumulated deposit on the membrane filter may mask or inhibit the growth of target organisms. The ideal range of the number of colonies that should be counted from a single membrane filter is 20 – 80 and the maximum number is approximately 100. Counts can be obtained from membrane filters containing more than 100 colonies provided that isolated colonies are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as an estimated count.

Where high numbers of organisms may be expected (for example, treated wastewater), serial ten-fold dilutions should be made to obtain a countable number of colonies on a membrane filter. For sand, sediments, and water with high turbidities, an appropriate MPN technique should be used, see method B.

## A6 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 assessments of risk 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, or when heated. Waste material containing sodium azide should, therefore, be discarded into drains with care, preferably through plastic pipes. Azide compounds may be decomposed and rendered safe with excess sodium nitrite, before disposal.

## A7 Apparatus

Standard laboratory equipment should be used which conforms to the performances 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 (at least 1 litre), made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

A7.2 Incubator capable of maintaining temperatures of 37 ± 1°C.

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 membranes of 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 formulations. 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 grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

### A8.1 *Vogel-Johnson agar*[3]

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| Mannitol | 10 g |
| Dipotassium phosphate | 5 g |
| Lithium chloride | 5 g |
| Glycine | 10 g |
| Phenol red | 25 mg |
| Potassium tellurite | 200 mg |
| Agar | 16 g |
| Water | 1 litre |

To prepare the medium dissolve the ingredients, except the potassium tellurite, in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving and cooling to approximately 50°C, the pH of the medium should be checked to confirm a pH of 6.9 ± 0.2. This medium may be stored in the dark at room temperature for up to one month. If used as prepared cool the molten medium to approximately 50°C prior to addition of potassium tellurite and pouring of plates. If the medium has been stored, heat the medium to 100°C to melt the medium. Potassium tellurite is prepared separately by dissolving 1 g in 100 ml (1 % m/v) of sterile water. Add 20 ml of a filter-sterilised 1 % m/v solution of potassium tellurite per litre of basal medium. Mix well. Pour the solution into sterile Petri dishes. Allow the medium to solidify. This medium may be stored in the range of 5 ± 3°C for up to one month, if protected from dehydration.

For samples from estuarine and marine environments, it may be that supplementation of the medium with 0.005 % of sodium azide may improve the specificity of the agar medium[5]. Sodium azide (50 mg) is added to the ingredients per litre of medium prior to autoclaving.

Similarly, for chlorinated waters, it may be that supplementation of the agar medium with 0.5 % w/v of pyruvate may improve the recovery of *Staphylococcus aureus*[6]. Sodium pyruvate (5 g/l) is added to the ingredients prior to autoclaving.

### A8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar, quarter strength Ringer's solution, maximum recovery diluent, Gram staining reagents, physiological saline solution and coagulase reagent.

## A9 Analytical procedure

### A9.1 *Sample preparation*

The volumes and dilutions of samples should be chosen so that the number of colonies to be counted on the membrane filter is as far as is practicable between 20 and 80, but not exceed 100 colonies. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent before filtration.

### A9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel. Holding the edge of the sterile membrane filter with sterile smooth-tipped forceps place it, if gridded grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample, or diluted sample, into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration 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 of Vogel-Johnson agar. 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.

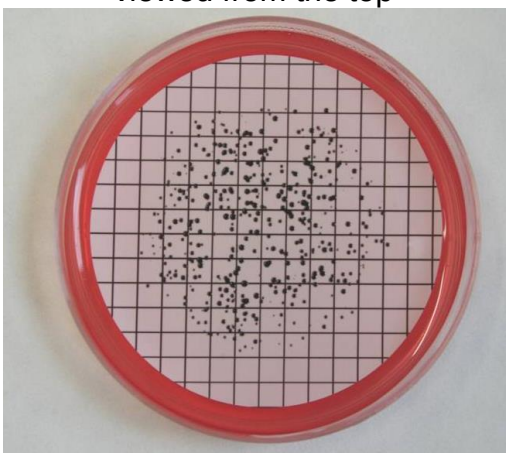
Incubate the Petri dishes at 37°C for 21 ± 3 hours.

A9.3 *Reading of results*

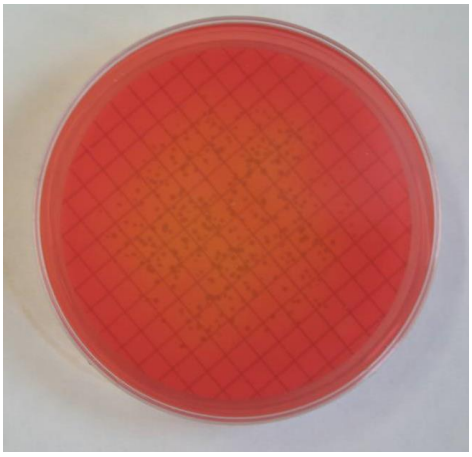
After incubation, examine the membrane filters under good light and count all black or dark grey, convex, shiny colonies which may have a yellow zone visible under the membrane filter (see Figure A1). These characteristic colonies are regarded as presumptive *Staphylococcus aureus.*

**Figure A1 Characteristic colonies of presumptive *Staphylococcus aureus* on Vogel-Johnson agar**

viewed from the top



viewed from the bottom



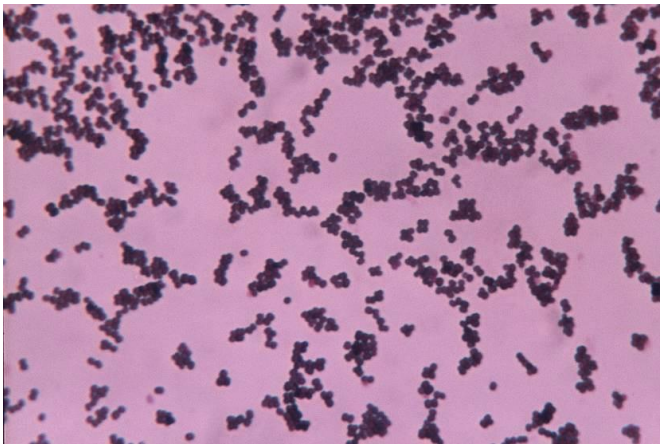
A9.4 *Confirmation tests*

Depending upon the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of black or dark grey colonies onto nutrient agar. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all black or dark grey colonies should be sub-cultured if fewer than ten are present. At least ten black or dark grey colonies should be sub-cultured if more than ten are present. A Petri dish containing nutrient agar is also streaked with a stock culture of *Staphylococcus aureus* and used as a positive control. The inoculated Petri dishes are inverted and incubated at 37°C for 21 ± 3 hours prior to conducting Gram staining[2, 7] and testing for coagulase production.

A9.4.1 *Gram stain*

Using a sterile loop, pick a discrete colony from the overnight nutrient agar culture and perform a Gram-stain[2, 7] . *Staphylococcus aureus* are observed as Gram-positive cocci resembling "bunches of grapes" (see Figure A2).

**Figure A2 Gram stain of *Staphylococcus aureus***



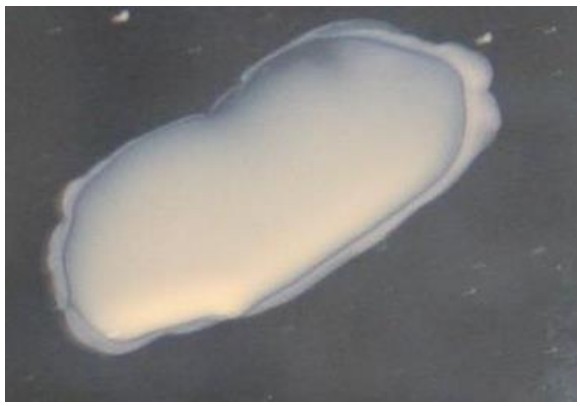
A9.4.2 *Coagulase test*

Possession of the enzyme coagulase which coagulates plasma is considered as an almost exclusive property of *Staphylococcus aureus.* The presence of coagulase may be detected (see Figure A3) with a rapid slide test using a coagulase reagent which consists of rabbit plasma. Using a clean microscope slide, emulsify a small portion of the colonies isolated from the Petri dish of nutrient agar in a drop (typically 0.02 ml) of water or physiological saline solution to produce a dense uniform suspension. Using a micro-loop or straight wire, add a small amount (typically 0.02 ml) of coagulase reagent, and mix well. *Staphylococcus aureus* will produce (typically within five seconds) a positive reaction, seen as visible clumping of the mixture. On each occasion that coagulase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (*Staphylococcus aureus*) and one species which is known to give a negative reaction (for example, *Staphylococcus warneri* or *Staphylococcus epidermidis*) (see Figure A3). Occasionally, auto-agglutination of colonies may occur at this stage which can mimic a genuine agglutination. If there is any doubt with the result a control test should also be included with only emulsified organism present.

**Figure A3 Coagulase test**

Positive coagulase reaction of *Staphylococcus aureus*

Negative coagulase reaction of *Staphylococcus epidermidis*



Commercial test kits for coagulase testing are available and should be used in accordance with manufacturer's instructions, following appropriate performance verification at the laboratory[2].

## A10 Calculations

A10.1 *Presumptive Staphylococcus aureus*

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

$$\text{Presumptive count per 100 ml} = \frac{\text{number of colonies counted on filter} \times 100 \times \text{DF}}{\text{volume of sample filtered (ml)}}$$

where DF is the dilution factor, if appropriate.

A10.2 *Confirmed Staphylococcus aureus*

Confirmed counts of *Staphylococcus aureus* are calculated by multiplying the number of presumptive *Staphylococcus aureus* by the proportion of the isolates that give typical morphology with Gram-staining and which are coagulase-positive.

## A11 Expression of results

Presumptive and confirmed *Staphylococcus aureus* are expressed in colony forming units per volume of sample.

## A12 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *Staphylococcus aureus*) and non-target bacteria (for example, *Escherichia coli*). Petri dishes should be incubated for 21 ± 3 hours at 37 °C. Further details are given elsewhere[2] in this series. Guidance on method quality control is given elsewhere[2] in this series.

## A13 References

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

2. 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. A modification of the tellurite-glycine media for the use in identification of *Staphylococcus aureus*, *Public Health Laboratory,* R A Vogel, and M J Johnson, 1960, **18**, 131-133.

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

5. Assessment of staphylococcus bacteria in Hawaii's marine recreational waters, *Water Science and Technology*, N Charoenca and R Fujioka, 1991, **27**, 283-289.

6. Evaluation of culture media for recovery of *Staphylococcus aureus* from swimming pools, *Applied and Environmental Microbiology*, R K Alico, and M F Dragonjac, 1986, **51**, 699-702.

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.

## B Method for the isolation and enumeration of *Staphylococcus aureus* by a presence-absence or multiple tube most probable number technique

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

### B1 Introduction

Staphylococci are widely distributed in the environment and may be isolated from foods, faeces, and the skin and mucous membranes of warm blooded animals. The organisms are opportunistic pathogens of man causing non-diarrhoeal diseases such as eye, ear, nose and skin infections, and as a consequence their isolation and enumeration may be required, particularly from swimming pools, spa pools and hydrotherapy pools. The significance of *Staphylococcus aureus* in recreational and other waters is described elsewhere[(1)] in this series.

### B2 Scope

The method is suitable for the examination of surface and saline waters, swimming pools, spa pools and hydrotherapy pools and primary and secondary wastewater effluents that contain high levels of sediments, or are highly turbid. In addition, solid samples may also be analysed.

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

### B3 Definitions

In the context of this method, following incubation in mannitol salt broth at 37°C, presumptive *Staphylococcus aureus* are defined as those bacteria which produce black or dark grey colonies on Vogel-Johnson agar after incubation at 37°C.

Confirmed *Staphylococcus aureus* are characteristic colonies obtained from Vogel-Johnson agar which when Gram-stained appear as Gram-positive cocci resembling "bunches of grapes" and which are coagulase-positive.

### B4 Principle

Mannitol salt broth is a high nutrient broth containing 7.5 % m/v sodium chloride. This makes the broth selective for presumptive pathogenic staphylococci. The medium contains mannitol as a fermentable carbohydrate and phenol red as an indicator of acidity. Organisms exhibiting characteristic growth in mannitol salt broth at 37°C for 21 ± 3 hours are sub-cultured onto the surface of Vogel-Johnson agar, i.e. an agar medium containing potassium tellurite and lithium chloride as selective agents, and mannitol as a fermentable carbohydrate. *Staphylococcus aureus* produce black or dark grey colonies as a result of the reduction of the tellurite to metallic tellurium.

## B5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

## B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate assessments of risk 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, or when heated. Waste material containing sodium azide should, therefore, be discarded into drains with care, preferably through plastic pipes. Azide compounds may be decomposed and rendered safe with excess sodium nitrite, before disposal.

## B7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, static temperature) are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume (at least 1 litre), made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

B7.2 Incubator capable of maintaining temperatures of 37 ± 1°C.

B7.3 Suitable bottle or test tube racks.

## B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

B8.1 *Single-strength mannitol salt broth*[4]

| | |
|---|---|
| Beef extract powder | 1 g |
| Peptone | 10 g |
| Mannitol | 75 g |
| Phenol red | 25 mg |
| Water | 1 litre |

Dissolve the ingredients in the water. To achieve this, it may be necessary to gently heat to 50°C. Dispense the resulting solution, in appropriate volumes, into suitable containers and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. The bottled medium may be stored in the dark at room temperature for up to one month.

Double-strength mannitol salt broth can be prepared using double the amounts of ingredients in 1 lire of water.

B8.2 *Vogel-Johnson agar*[5]

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| Mannitol | 0 g |
| Dipotassium phosphate | 5 g |
| Lithium chloride | 5 g |
| Glycine | 10 g |
| Phenol red | 25 mg |
| Potassium tellurite | 16 g |
| Water | 1 litre |

To prepare the medium dissolve the ingredients, except the potassium tellurite, in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving and cooling to approximately 50°C, the pH of the medium should be checked to confirm a pH of 6.9 ± 0.2. This medium may be stored in the dark at room temperature for up to one month. If used as prepared cool the molten medium to approximately 50°C prior to addition of potassium tellurite and pouring of plates. If the medium has been stored, heat the medium to 100°C to melt the medium. Potassium tellurite is prepared separately by dissolving 1 g in 100 ml (1 % m/v) of sterile water. Add 20 ml of a filter-sterilised 1 % m/v solution of potassium tellurite per litre of basal medium. Mix well. Pour the solution into sterile Petri dishes. Allow the medium to solidify. This medium may be stored in the range of 5 ± 3°C for up to one month, if protected from dehydration.

For samples from estuarine and marine environments, it may be that supplementation of the medium with 0.005 % of sodium azide may improve the specificity of the agar medium[6]. Sodium azide (50 mg) is added to the ingredients prior to autoclaving.

Similarly, for chlorinated waters, it may be that supplementation of the agar medium with 0.5 % of pyruvate might improve the recovery of *Staphylococcus aureus*[7]. Sodium pyruvate (5 g) is added to the ingredients prior to autoclaving.

B8.3 *Filter-aid*[8]

| | |
|---|---|
| Diatomaceous earth | 1 g (approximately) |
| Water | 15 ml |

Weigh out appropriate amounts of filter-aid into suitable bottles, add the water and cap. Sterilise by autoclaving at 121°C for 15 minutes. The sterilised filter-aid may be stored in the dark at room temperature for up to 12 months.

B8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar, quarter strength Ringer's solution, maximum recovery diluent, Gram staining reagents, physiological saline solution and coagulase reagent.

## B9 Analytical procedure

B9.1 *Sample preparation*

B9.1.1 *Surface waters, marine waters, swimming pool and similar waters*

As the numbers of *Staphylococcus aureus* in some surface waters, marine and swimming pool waters waters, are likely to be absent or present in low numbers, it is recommended that for presence-absence determinations a sample volume of at least 1000 ml is examined.

For the membrane filtration multiple tube technique, typically, an 11-tube series can be used requiring a sample volume of 1050 ml, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 1 x 500 ml and 5 x 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes of double-strength mannitol salt broth. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single-strength mannitol salt broth. Turbid waters, unsuitable for direct membrane filtration, may be filtered using filter aid.

B9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in B9.1.1 although several membrane filters may be required for presence-absence determinations. A sample volume of at least 100 ml may need to be examined.

The volumes may be reduced and volumes of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml be used. The 1 x 50 ml and 5 x 10 ml volumes can be membrane filtered or added to equal volumes of double-strength mannitol salt broth. To represent smaller volumes of samples, a 1:10 dilution of the sample, for example 1 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate. Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength mannitol salt broth.

B9.1.3 *Untreated wastewater*

For presence-absence determinations, 100 ml of untreated wastewater sample may

be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration. For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 x 50 ml and 5 x 10 ml volumes can be filtered or added to equal volumes of double-strength mannitol salt broth. The 1 ml volumes can be added to 9 ml of single-strength mannitol salt broth. To represent smaller volumes, for example 0.1 ml and 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample, may be appropriate. Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength mannitol salt broth.

B9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence-absence determinations by weighing, for example 10 g of sample into an appropriate volume (typical 100 ml) of single-strength mannitol salt broth. For the multiple tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g quantities of sample into appropriate volumes (typical 450 ml, 5 x 100 ml and 5 x 10 ml respectively) of single-strength mannitol salt broth. For smaller quantities, for example 100 mg, these may be added directly to 10 ml of single-strength mannitol salt broth.

B9.2 Sample processing

B9.2.1 *Membrane presence-absence or filtration multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter onto the porous disc of the filter base. If a gridded membrane filter is used, place grid-side upwards. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing, typically, 10 - 15 ml of single-strength mannitol salt broth, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single-strength mannitol salt broth. The largest single volume of sample may require more than one membrane filter and, if so, all filters used for this volume should be transferred to the bottle or tube of single-strength mannitol salt broth. Ensure that all membrane filters are fully submerged.

The funnel 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 boiling provided that the smallest volume of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then 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 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 disinfected 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.

B9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to single-strength mannitol salt broth. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml.

For presence-absence determinations, place the culture vessels in the incubator. For a most probable number test, re-suspend the filter aid in the single-strength mannitol salt broth and dispense in a multiple tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes can be inoculated into 9 ml of sterile single-strength mannitol salt broth.

B9.2.3 *Direct inoculation*

Where the numbers of *Staphylococcus aureus* in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into equal volumes of double strength mannitol salt broth. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single-strength mannitol salt broth.

B9.2.4 *Sediment and sand*

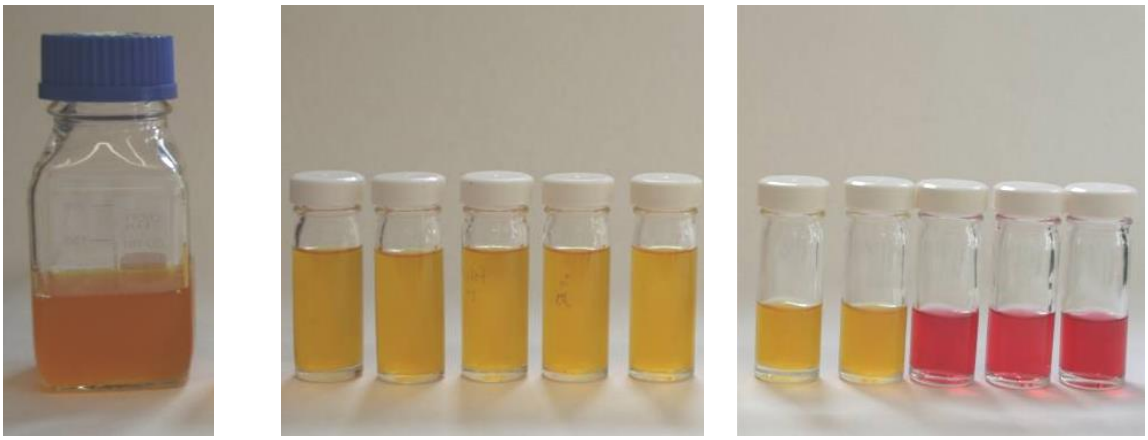
Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence absence determinations or 1 x 5 g, into 90 ml, and 5 x 1 g and 5 x 0.1 g into 9 ml of single-strength mannitol salt broth for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single-strength mannitol salt broth.

B9.3 *Enrichment and subculture to selective agar*

The mannitol salt broth and membrane filters, and if appropriate filter-aid or solid sample material, is mixed thoroughly, each tube or bottle capped or sealed and incubated at 37°C for 21 ± 3 hours. After incubation, the tubes or bottles are examined for growth (demonstrated by acid production and the presence of yellow colouration). (see Figure B1). All tubes or bottles that exhibit characteristic growth within the medium are retained for confirmatory testing.

**Figure B1 MPN series of bottles inoculated with wastewater exhibiting growth (yellow tubes, i.e. positive) and no growth (red tubes, i.e. negative) for presumptive *Staphylococcus aureus***

| 1 x 50 ml, plus equal volume of double-strength medium | 5 x 10 ml, plus equal volumes of double-strength medium | 5 x 1 ml, plus 9 ml of single-strength medium |
|---|---|---|



Bottles that exhibit growth within the medium are indicated by yellow colouration, regard these as positive. Bottles that exhibit no growth within the medium are indicated by red colouration, regard these as negative.

B9.4 *Reading of results*

The number of tubes or bottles for each series of sample is recorded where a positive reaction is given, as demonstrated by growth within the medium (see Figure B1). When dilutions of sample have been used, a consecutive series of volumes is chosen whereby some of the tubes or bottles show a positive reaction and some show a negative reaction. From the results, the MPN of presumptive *Staphylococcus aureus* in the sample is determined from probability tables, see appendix B1. Any bottles exhibiting characteristic growth, i.e. showing acid, indicated by a change in the colour of the medium from red to yellow, should be sub-cultured to Petri dishes containing Vogel-Johnson agar and incubated at 37°C for 21 ± 3 hours.

B9.5 *Confirmation tests*

Depending upon the intended purpose of the analysis and the required accuracy, sub-culture one or two black or dark grey colonies from the Vogel-Johnson agar onto nutrient agar. A Petri dish containing nutrient agar is also streaked with a stock culture of *Staphylococcus aureus* and used as a positive control. The inoculated nutrient agar Petri dishes are inverted and incubated at 37 ± 1°C for 21 ± 3 hours prior to conducting Gram staining[2, 9] and testing for coagulase production.

B9.5.1 *Gram stain*

Using a sterile loop, pick a discrete colony from the overnight nutrient agar culture and perform a Gram-stain[2, 9]. *Staphylococcus aureus* are observed as Gram-positive cocci resembling "bunches of grapes" (see Figure B3).

**Figure B2 Characteristic colonies of presumptive *Staphylococcus aureus* on Vogel-Johnson agar sub-cultured from mannitol salt broth**

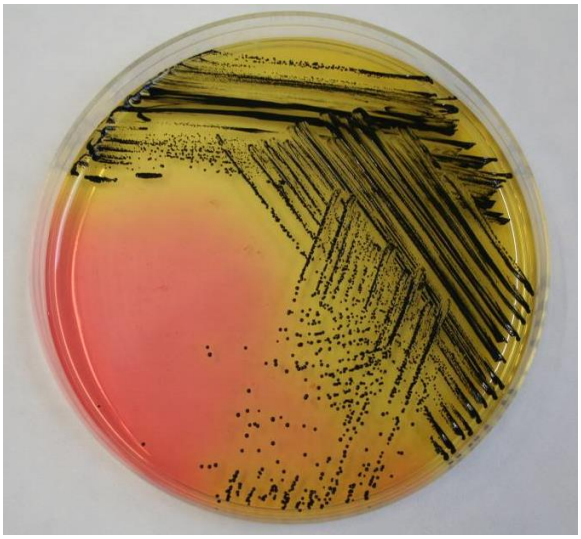
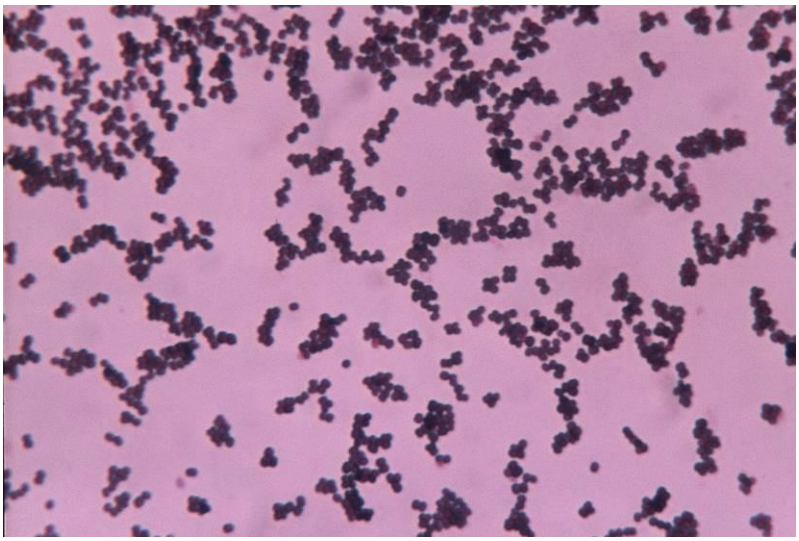


**Figure B3 Gram stain of *Staphylococcus aureus***



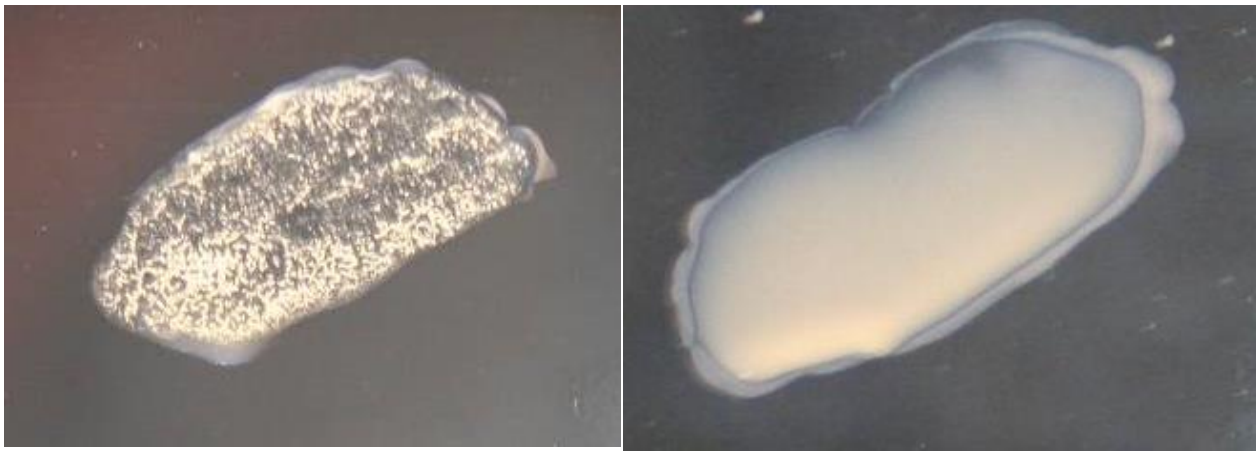
B9.5.2 *Coagulase test*

Possession of the enzyme coagulase which coagulates plasma is considered as an almost exclusive property of *Staphylococcus aureus.* The presence of coagulase may be detected using a rapid slide test using a coagulase reagent which consists of rabbit plasma. Using a clean microscope slide, emulsify a small portion of the colonies isolated from the Petri dish of nutrient agar in a drop (typically 0.02 ml) of water or physiological saline solution to produce a dense uniform suspension. Using a micro-loop or straight wire, add a small amount (typically 0.02 ml) of coagulase reagent, and mix well. *Staphylococcus aureus* will produce (typically within five seconds) a positive reaction, seen as visible clumping of the mixture. On each occasion that coagulase 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 which is known to give a negative reaction (for example, *Staphylococcus warneri* or *Staphylococcus epidermidis*) (see Figure B4). Occasionally, auto-agglutination of colonies may occur at this stage which can emulate a genuine agglutination. If there is

any doubt with the result a control test should also be included with only emulsified organism present.

**Figure B4 Coagulase test**

| Positive coagulase reaction of *Staphylococcus aureus* | Negative coagulase reaction of *Staphylococcus epidermidis* |
|---|---|



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

## B10 Calculations

B10.1 *Presumptive Staphylococcus aureus*

The number of mannitol salt broth medium tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in appendix B1, the MPN of presumptive *Staphylococcus aureus* present in 100 ml of sample is determined. For example, if in an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 1, 4 and 2 respectively, then, from Table B2, the MPN is 21 organisms per 100 ml.

B10.2 *Confirmed Staphylococcus aureus*

Confirmed counts of *Staphylococcus aureus* are calculated by multiplying the number of presumptive *Staphylococcus aureus* by the proportion of the isolates that give typical morphology with Gram-staining and which are coagulase-positive.

## B11 Expression of results

Presumptive and confirmed *Staphylococcus aureus* are expressed in colony forming units per volume or weight of sample.

## B12 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *Staphylococcus aureus*) and non-target bacteria (for example, *Escherichia coli*). Petri dishes should be incubated for 21 ± 3 hours at 37 °C. Further details are given elsewhere(2) in this series. Guidance on method quality control is given elsewhere(2) in this series.

## B13 References


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

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

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

4. The reliability of bromthymol-blue lactose agar and bacto phenol-red mannitol agar for the isolation of pathogenic staphylococci, *Journal of Bacteriology,* G H Chapman, 1944, **48,** 555-557.

5. A modification of the tellurite-glycine media for the use in identification of *Staphylococcus aureus*, *Public Health Laboratory,* R A Vogel, and M J Johnson, 1960, **18**, 131-133.

6. Assessment of staphylococcus bacteria in Hawaii's marine recreational waters, *Water Science and Technology*, N Charoenca and R Fujioka, 1991, **27**, 283-289.

7. Evaluation of culture media for recovery of *Staphylococcus aureus* from swimming pools, *Applied and Environmental Microbiology*, R K Alico, and M F Dragonjac, 1986, **51**, 699-702.

8. Concentration technique for demonstrating small amounts of bacteria in tap water, *Acta Pathologica et Microbiologica Scandinavica*, E Hammarstrom and V Ljutov, 1954, **35**, 365 - 369.

9. *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.

## Appendix B1 Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 100 ml of sample. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values. Approximate 95 % confidence intervals, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published[(1)]. A procedure for estimating these confidence intervals for other dilution series has also been published[(2)]. These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source[(3)]. The MPR in tables B1 - B3 illustrates those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to tubes of medium.

Table B1 gives the MPN (and where applicable the MPR) for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample. Similarly table B2 gives the MPN (and where applicable the MPR) for an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Table B3 shows data for a 15-tube series of 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of samples but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.1 ml tubes would not be expected if all of the 10 ml and 1 ml tubes were negative. Hence, MPN and MPR values for a combination of results like for instance 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct[(1, 4, 5)]. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic substances may have been present.

### Calculation of MPN

The number of positive reactions for each set of tubes is recorded and, from the relevant table, the MPN of organisms present in 100 ml of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in table B4.

(i) Use only three consecutive sets of dilutions for calculating the MPN.

(ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in table B4).

(iii) If less than three sets of dilutions give positive results, begin with the set containing the largest volume of sample (see example (d) in table B4).

(iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in table B4).

**Table B1 MPN and MPR per 100 ml of sample for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample**

| Number of tubes giving a positive reaction | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|
| 1 x 50 ml | 5 x 10 ml | | |
| 0 | 0 | None found | |
| 0 | 1 | 1 | |
| 0 | 2 | 2 | |
| 0 | 3 | 3 | |
| 0 | 4 | 4 | 4-5 |
| 0 | 5 | 6 | |
| 1 | 0 | 1 | |
| 1 | 1 | 2 | |
| 1 | 2 | 5 | 4-5 |
| 1 | 3 | 9 | 8-10 |
| 1 | 4 | 15 | 13-18 |
| 1 | 5 | >18** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 18.

**Table B2 MPN and MPR per 100 ml of sample for an 11-tube series of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample**

| Number of tubes giving a positive reaction | | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|---|
| 1 x 50 ml | 5 x 10 ml | 5 x 1 ml | | |
| 0 | 0 | 0 | None found | |
| 0 | 0 | 1 | 1 | |
| 0 | 1 | 0 | 1 | |
| 0 | 1 | 1 | 2 | |
| 0 | 2 | 0 | 2 | |
| 0 | 2 | 1 | 3 | |
| 0 | 3 | 0 | 3 | |
| 1 | 0 | 0 | 1 | |
| 1 | 0 | 1 | 2 | |
| 1 | 1 | 0 | 2 | |
| 1 | 1 | 1 | 4 | |
| 1 | 1 | 2 | 6 | |
| 1 | 2 | 0 | 4 | 4-5 |
| 1 | 2 | 1 | 7 | 6-7 |
| 1 | 2 | 2 | 9 | 9-10 |
| 1 | 3 | 0 | 8 | 7-9 |
| 1 | 3 | 1 | 10 | 10-11 |
| 1 | 3 | 2 | 13 | 12-13 |
| 1 | 3 | 3 | 17 | 15-18 |
| 1 | 4 | 0 | 12 | 11-14 |
| 1 | 4 | 1 | 16 | 15-19 |
| 1 | 4 | 2 | 21 | 19-24 |
| 1 | 4 | 3 | 27 | 24-30 |
| 1 | 4 | 4 | 33 | 30-38 |
| 1 | 5 | 0 | 23 | 20-27 |
| 1 | 5 | 1 | 33 | 29-40 |
| 1 | 5 | 2 | 53 | 44-65 |
| 1 | 5 | 3 | 91 | 75-110 |
| 1 | 5 | 4 | 160 | 134-190 |
| 1 | 5 | 5 | >180** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 180.

**Table B3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample**

| Number of tubes giving a positive reaction | | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|---|
| 5 x 10 ml | 5 x 1 ml | 5 x 0.1 ml | | |
| 0 | 0 | 0 | None found | |
| 0 | 0 | 1 | 2 | |
| 0 | 1 | 0 | 2 | |
| 1 | 0 | 0 | 2 | |
| 1 | 0 | 1 | 4 | |
| 1 | 1 | 0 | 4 | |
| 2 | 2 | 0 | 4 | |
| 2 | 0 | 1 | 5 | |
| 2 | 1 | 0 | 5 | |
| 2 | 1 | 1 | 7 | |
| 2 | 2 | 0 | 7 | 7-9 |
| 2 | 3 | 0 | 11 | |
| 3 | 0 | 0 | 7 | |
| 3 | 0 | 1 | 9 | |
| 3 | 1 | 0 | 9 | |
| 3 | 1 | 1 | 13 | |
| 3 | 2 | 0 | 13 | |
| 3 | 2 | 1 | 16 | 14-16 |
| 3 | 3 | 0 | 16 | 14-16 |
| 4 | 0 | 0 | 11 | 11-13 |
| 4 | 0 | 1 | 14 | 14-16 |
| 4 | 1 | 0 | 16 | 14-16 |
| 4 | 1 | 1 | 20 | 18-20 |
| 4 | 2 | 0 | 20 | 18-22 |
| 4 | 2 | 1 | 25 | 23-27 |
| 4 | 3 | 0 | 25 | 23-27 |
| 4 | 3 | 1 | 31 | 29-34 |
| 4 | 4 | 0 | 32 | 29-34 |
| 4 | 4 | 1 | 38 | 34-41 |
| 5 | 0 | 0 | 22 | 20-23 |
| 5 | 0 | 1 | 29 | 25-34 |
| 5 | 0 | 2 | 41 | 36-50 |
| 5 | 1 | 0 | 31 | 27-36 |
| 5 | 1 | 1 | 43 | 36-50 |
| 5 | 1 | 2 | 60 | 50-70 |
| 5 | 1 | 3 | 85 | 70-95 |
| 5 | 2 | 0 | 50 | 40-55 |
| 5 | 2 | 1 | 70 | 60-80 |
| 5 | 2 | 2 | 95 | 80-110 |
| 5 | 2 | 3 | 120 | 105-135 |
| 5 | 3 | 0 | 75 | 65-90 |
| 5 | 3 | 1 | 110 | 90-125 |
| 5 | 3 | 2 | 140 | 120-160 |
| 5 | 3 | 3 | 175 | 155-200 |
| 5 | 3 | 4 | 210 | 185-240 |
| 5 | 4 | 0 | 130 | 110-150 |
| 5 | 4 | 1 | 170 | 150-200 |
| 5 | 4 | 2 | 220 | 190-250 |
| 5 | 4 | 3 | 280 | 240-320 |
| 5 | 4 | 4 | 345 | 300-390 |
| 5 | 5 | 0 | 240 | 200-280 |
| 5 | 5 | 1 | 350 | 290-420 |
| 5 | 5 | 2 | 540 | 450-600 |
| 5 | 5 | 3 | 910 | 750-1100 |
| 5 | 5 | 4 | 1600 | 1350-1900 |
| 5 | 5 | 5 | >1800** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 1800.

**Table B4 Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions***

| Example in text | Volume of sample (ml) | | | | | MPN per 100 ml |
|---|---|---|---|---|---|---|
| | 10 | 1 | 0.1 | 0.01 | 0.001 | |
| (a) | ***5*** | ***3*** | ***2*** | 0 | | 140 |
| (b) | 5 | ***5*** | ***3*** | ***2*** | 0 | 1400 |
| (c) | 5 | ***5*** | ***2*** | ***0*** | 0 | 500 |
| (d) | ***3*** | ***1*** | ***0*** | 0 | | 9 |
| (e) | ***0*** | ***1*** | ***0*** | 0 | | 2 |

- Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

**References**


1. The range of variation of the most probable number of organisms estimated by the dilution method, *Indian Journal of Medical Research*, S Swaroop, 1951, **39**, 107-134.

2. Automated statistical analysis of microbial enumeration by dilution series, *Journal of Applied Bacteriology*, M A Hurley & M E Roscoe, 1983, **55**, 159-164.

3. Estimated numbers of bacteria in samples from non-homogeneous bodies of water: how should MPN and membrane filtration results be reported? *Journal of Applied Bacteriology*, H E Tillett & R Coleman, 1985, **59**, 381-388.

4. How probable is the most probable number? *Journal of the American Waterworks Association*, R L Woodward, 1957, **49**, 1060-1068.

5. The probability of most probable numbers. *European Journal of Applied Microbiology*, J C de Man, 1975, **1**, 67-78.

## Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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

## Standing Committee of Analysts

## Members assisting with this method

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

| | |
|---|---|
| Peter Boyd | Formerly Public Health England |
| Simon Cole | Wessex Water |
| David Gaskell | United Utilities |
| Phil Holmes | Formerly Severn Trent Water |
| Malcolm Morgan | SCA Strategic Board |
| David Sartory | SWM Consulting |
| Martin Walters | Environment Agency |
| John Watkins | CREH *Analytical* Limited |
| John Watson | South West Water |
| David Westwood | Formerly Environment Agency |

Grateful acknowledgement is made to John Watkins (CREH *Analytical*) for providing colour photographs.