



ENVIRONMENT AGENCY

**The Microbiology of Drinking Water (2002) - Part 8 - Methods for the
isolation and enumeration of *Aeromonas* and
Pseudomonas aeruginosa by membrane filtration**

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2002) - Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration

Methods for the Examination of Waters and Associated Materials

This booklet contains two methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas* by membrane filtration.

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

Part 1 - Water quality and public health

Part 2 - Practices and procedures for sampling

Part 3 - Practices and procedures for laboratories

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

Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration

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

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

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

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

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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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 these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods is available from the Secretary.

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

Dr D Westwood
Secretary

January 2002

Warning to users

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

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1999 (SI 1999/437). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

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

A The isolation and enumeration of mesophilic *Aeromonas* species by membrane filtration

A1 Introduction

Bacteria of the genus *Aeromonas* are commonly found in fresh and estuarine waters and sewage. Mesophilic species are frequently isolated from drinking water, particularly in the summer months, and are often associated with biofilm development and after-growth problems. At present, all mesophilic *Aeromonas* species may be considered equally significant and for practical purposes it is not necessary to identify *Aeromonas* species beyond the level of genus.

Mesophilic *Aeromonas* may colonise, or infect, wounds contaminated with water, and can cause septicaemia in immuno-compromised individuals. The organisms have been incriminated as a cause of diarrhoea and wound infections related to contact with soil and water have been reported.

There is some evidence to suggest that environmental strains are non-pathogenic and that pathogenic strains produce cytotoxins. This can be demonstrated using classical cytotoxicity tests, or β -haemolysis of horse-blood on horse-blood agar, or other biochemical tests. The significance of *Aeromonas* in water treatment and supply are described elsewhere⁽¹⁾ in this series.

A2 Scope

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

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

A3 Definitions

In the context of this method, *Aeromonas* species form characteristic yellow or yellow/green colonies on ampicillin dextrin agar. The bacteria are oxidative and fermentative in O/F medium⁽³⁾, oxidase-positive and resistant to the vibriostatic agent 0129 phosphate (2,4-diamino-6,7-diisopropylpteridine phosphate).

A4 Principle

Bacteria are isolated on membrane filters placed on an agar medium containing ampicillin as a selective agent, dextrin as the fermentable carbohydrate and bromothymol blue as an indicator of acidity.

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. Accumulated deposit on the membrane filter may mask or inhibit the growth of target organisms. High numbers of competing organisms (for

example, species of *Vibrio*) may inhibit the growth or mask the detection of target organisms. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

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 risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere⁽²⁾ in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere⁽²⁾. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

A7.1 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 Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent). A solution of tetra-sodium ethylenediaminetetraacetic acid or tri-sodium nitrilotriacetate, to achieve a final concentration in the sample of 50 mg/l (for example, 0.1 ml of a 50 g/l solution for every 100 ml of sample) may also be added to the sample bottle before sterilisation if high levels of copper are envisaged in the sample⁽⁵⁾.

A7.2 Incubator capable of maintaining a temperature of 30 ± 1 °C.

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

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

A7.5 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in the method. Variations in the preparation and storage of media should also be verified.

A8.1 *Ampicillin dextrin agar*⁽⁶⁾

Tryptose	5 g
Dextrin	10 g
Yeast extract	2 g
Sodium chloride	3 g
Potassium chloride	2 g

Magnesium sulphate heptahydrate	200 mg
Iron(III) chloride	100 mg
Bromothymol blue (1% m/v aqueous solution)	8 ml
Agar	15 g
Sodium desoxycholate	100 mg
Ampicillin	10 mg
Distilled, deionised or similar grade water	1 litre

Dissolve all the ingredients, except the agar, ampicillin and desoxycholate in the water. Adjust the pH to 8.0 ± 0.2 . Add the agar and dissolve. This will require heating the solution to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilise by autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 8.0 ± 0.2 . Cool the molten medium to approximately $50\text{ }^{\circ}\text{C}$ and add 10 ml of a freshly prepared aqueous filter-sterilised solution of ampicillin (containing 1 mg/ml) and 10 ml of an aqueous filter-sterilised solution of desoxycholate (containing 10 mg/ml) per litre of medium. Mix thoroughly, pour into sterile Petri dishes and allow the medium to solidify. The agar Petri dishes should be stored at between $2 - 8\text{ }^{\circ}\text{C}$, protected against dehydration, and used within seven days. Alternatively, the medium without ampicillin and desoxycholate can be stored at room temperature and used within one month if protected against dehydration.

A8.2. *Hugh and Leifson's O/F medium*⁽³⁾

Peptone	2 g
Sodium chloride	5 g
Glucose	10 g
Dipotassium hydrogen phosphate	300 mg
Bromothymol blue (1 % m/v aqueous solution)	3 ml
Agar	3 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Adjust the pH to 7.1 ± 0.2 and dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilise by autoclaving at $115\text{ }^{\circ}\text{C}$ for 10 minutes. After autoclaving, the pH of the base medium should be checked to confirm a pH of 7.1 ± 0.2 . The prepared medium may be stored between $2 - 8\text{ }^{\circ}\text{C}$, protected against dehydration. Use within one month.

Alternatively, the base medium without the glucose can be stored in the dark at room temperature and used within one month if protected against dehydration.

A8.3 *Other media*

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

A9 Analytical procedure

A9.1 Sample preparation

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For treated 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 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 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 so that as little air as possible is drawn through the membrane filter.

Remove the funnel and transfer the membrane filter carefully to a Petri dish of ampicillin dextrin agar. 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 boiling provided that the smallest volume or highest dilution of sample is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade 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 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 30 °C for 20 - 24 hours.

A9.3 *Reading of results*

After incubation, examine the filters for typical colonies that are 2 - 3 mm in diameter, smooth with an entire edge, and yellow or yellow with a greenish-yellow periphery. Colonies that are completely blue or white, and translucent in appearance should not be counted. Some species of *Vibrio* will grow on ampicillin dextrin agar producing yellow colonies.

A9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow colonies or those yellow with a greenish-yellow periphery. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present. At least ten colonies should be sub-cultured if more than ten are present.

A9.4.1 *Oxidase test*

Subculture suspect colonies to nutrient agar and incubate at 30 °C for 20 - 24 hours.

Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the nutrient agar onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction. *Aeromonas* species are oxidase-positive.

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

On each occasion that oxidase reagent is used, conduct control tests with organisms of which one species is known to give a positive reaction (for example *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example *Escherichia coli*).

A9.4.2 *Hugh and Leifson's O/F test*

Prior to inoculation, tubes containing O/F medium should be brought to room temperature before use. With a straight wire, subculture each suspect colony (or growth from nutrient agar) to two O/F tubes. Inoculate to the bottom of each tube. Cover the medium in one of the O/F tubes with a small amount of sterile mineral oil. Incubate both tubes at 30 °C for 20 - 24 hours. Alternatively, incubate one tube (without mineral oil) in an anaerobe jar, and another tube (again, without mineral oil) aerobically, both at 30 °C for 20 - 24 hours.

At the same time inoculate pairs of tubes with organisms known to give a positive fermentation reaction (for example *Aeromonas hydrophila*) and one species known to give an oxidative only reaction (for example *Pseudomonas aeruginosa*).

Examine the O/F tubes for growth and oxidative or fermentative reactions. A fermentative reaction will show acid production in both tubes. An oxidative reaction should show a colour change due to acid production in the tube incubated aerobically. There should be no acid production in the tube covered with the mineral oil or the tube incubated anaerobically.

A9.4.3 0129 vibriostatic test

In order to distinguish between *Aeromonas* and *Vibrio* species, a vibriostatic (0129) test may be performed.

Using a sterile loop, transfer a colony from the nutrient agar and inoculate a Petri dish of freshly dried nutrient agar. Using a sterile swab, smear the colony over the agar surface, rotate the plate 90 ° and smear again. Using sterile forceps, place a diagnostic disc impregnated with 150 µg of the vibriostatic agent in the centre of the agar surface.

Invert the Petri dish and incubate at 30 °C overnight. Resistance to 0129 (ie, negative sensitivity allowing growth on the plate right up to the disc) should be considered as indicative for *Aeromonas* species. Petri dishes displaying zones of non-growth next to the disc (ie, positive sensitivity) should be considered indicative of *Vibrio* species.

Confirmation of isolates may also be carried using commercially available biochemical tests, following appropriate performance verification at the laboratory.

A9.4.4 Speciation of isolates

Mesophilic *Aeromonas* isolates may be phenotypically speciated using the following simplified biochemical testing scheme⁽⁷⁾:-

	Gas from glucose	Hydrolysis of aesculin
<i>Aeromonas hydrophila</i>	+	+
<i>Aeromonas caviae</i>	-	+
<i>Aeromonas sobria</i>	+	-
Unidentified <i>Aeromonas</i>	-	-

A10 Calculations

A10.1 Presumptive *Aeromonas*

The number of presumptive *Aeromonas* bacteria is generally expressed as the number of colonies per 100 ml. Calculate the presumptive count as follows:

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

A10.2 Confirmed *Aeromonas*

Confirmed counts of *Aeromonas* species are calculated by multiplying the number of presumptive *Aeromonas* by the proportion of the isolates that give a fermentative reaction in the O/F test, are oxidase-positive and are resistant to the vibriostatic agent 0129.

A11 Expression of results

Presumptive and confirmed *Aeromonas* species are expressed in colony forming units per volume of sample.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Aeromonas hydrophila*) and non-target bacteria (for example *Escherichia coli*). Petri dishes should be incubated at 30 °C for 24 hours. Further details are given elsewhere⁽²⁾ in this series.

A13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
3. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *Journal of Bacteriology*, Hugh, R. & Leifson, E., 1953, **66**, 24-26.
4. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
5. Prevention of toxicity of metal ions to *Aeromonas* and other bacteria in drinking-water samples using nitrilotriacetic acid (NTA) instead of ethylenediaminetetraacetic acid (EDTA). *Letters in Applied Microbiology*, Schets, F. M. & Medema, G. J., 1993, **16**, 75-76.
6. Ampicillin-dextrin agar medium for the enumeration of *Aeromonas* species in water by membrane filtration. *Journal of Applied Bacteriology*, Havelaar, A. H., During, M. & Versteegh, J. F. M., 1987, **62**, 279-287.
7. The presence of *Aeromonas* in drinking water supplies in the Netherlands. *Zentralblatt für Hygiene*, Havelaar, A. H., Versteegh, J. F. M. & During, M., 1990, **190**, 236-256.

B The isolation and enumeration of *Pseudomonas aeruginosa* by Membrane Filtration

B1 Introduction

Pseudomonas aeruginosa are environmental bacteria commonly found in soil and on plants. The organisms are able to grow in waters containing very low levels of nutrients and should be absent in bottled waters or in water used in the manufacture of food, drink and pharmaceutical products and in hospitals. It is frequently present, in small numbers, in the normal intestinal flora of humans and animals but should not be used as an indicator of faecal pollution.

Pseudomonas aeruginosa are opportunistic pathogens, particularly in humans who are immuno-compromised. Principal infections include septicaemia, skin, burn, respiratory, urinary tract and ear infections. Large numbers growing in polluted waters, swimming pool waters or spa pool waters may, following immersion, produce ear infections or a follicular dermatitis. The organism is important because of its antibiotic resistance. The significance of *Pseudomonas aeruginosa* in water treatment and supply are described elsewhere⁽¹⁾ in this series.

B2 Scope

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

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

B3 Definitions

Pseudomonas aeruginosa are Gram-negative, oxidase-positive bacteria which, in the context of this method, usually produce pyocyanin and fluorescein, and are capable of hydrolysing casein.

B4 Principle

Bacteria are isolated on membrane filters placed on a solid medium containing magnesium chloride and potassium sulphate to enhance pigment production. The medium is made selective by the addition of cetyl trimethylammonium bromide and nalidixic acid. *Pseudomonas aeruginosa* usually produce characteristic blue-green or brown coloured colonies. Confirmation of isolates is by subculture to milk agar to demonstrate hydrolysis of casein.

B5 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. Accumulated deposit on the membrane filter may mask or inhibit the growth of target organisms. High numbers of competing organisms may inhibit the growth or detection of target organisms.

B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations⁽³⁾ and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere⁽²⁾ in this series. When an ultra-violet (UV) lamp is used it is advised that gloves and either goggles or a face shield suitable for use with appropriate UV emitting sources are worn.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere⁽²⁾. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

- B7.1 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 Na₂S₂O₃.5H₂O per 100 ml of sample, or equivalent).
- B7.2 Incubator capable of maintaining a temperature of 37 ± 1 °C.
- B7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.
- B7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based, 0.45 µm nominal pore size.
- B7.5 Smooth-tipped forceps.
- B7.6 An ultraviolet lamp capable of an output of 360 ± 20 nm

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in the method. Variations in the preparation and storage of media should also be verified.

B8.1 *Pseudomonas* agar

Gelatin peptone	16 g
Casein hydrolysate	10 g
Potassium sulphate	10 g
Magnesium chloride	1.4 g
Glycerol	10 ml
Cetyl trimethylammonium bromide	200 mg
Nalidixic acid, sodium salt	15 mg
Agar	11 g
Distilled, deionised or similar grade water	1 litre

Dissolve the solid ingredients, except the cetyl trimethylammonium bromide and nalidixic acid, in water. To dissolve the ingredients it will be necessary to heat to boiling. Add 10 ml of glycerol and mix well. 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 base medium should be checked to confirm a pH of 7.1 ± 0.2 . Cool the molten base medium to approximately 50 °C and add the cetyl trimethylammonium bromide and nalidixic acid as filter-sterilised aqueous sterile solutions to give final concentrations of 200 mg/l and 15 mg/l respectively. Mix thoroughly, and dispense into sterile Petri dishes. Allow the complete medium to solidify, store at between 2 - 8 °C, protected from dehydration, and use within one month. Alternatively, the base medium can be stored in the dark at room temperature and used within one month.

B8.2 *Milk agar with cetyl trimethylammonium bromide*⁽⁴⁾

B8.2.1 *Yeast extract broth*

Bacteriological peptone	10 g
Yeast extract	3 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Adjust the pH to 7.3 ± 0.2 and sterilise by autoclaving at 121 °C for 15 minutes.

B8.2.2 *Milk agar with cetyl trimethylammonium bromide - complete medium*

Skimmed-milk powder	100 g
Yeast extract broth (from B8.2.1)	250 ml
Agar	15 g
Cetyl trimethylammonium bromide	300 mg
Distilled, deionised or similar grade water	750 ml

Add the cetyl trimethylammonium bromide and agar to 250 ml of sterile yeast extract broth, mix well and steam to dissolve. Thoroughly mix the skimmed-milk powder with 750 ml of water. Autoclave the individual solutions separately at 121 °C for 5 minutes. Cool to approximately 50 - 55 °C, add the skimmed-milk powder solution to the agar solution, mix thoroughly and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8 °C, protected against dehydration, and use within one month.

B8.3 *Other media*

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

B9 Analytical procedure

B9.1 Sample preparation

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

B9.2 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 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 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 water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered so that as little air as possible is drawn through the membrane filter.

Remove the funnel and transfer the membrane filter carefully to a Petri dish of *Pseudomonas* agar. 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 boiling provided that the smallest volume or highest dilution of sample is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade 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 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 *Pseudomonas* agar Petri dishes at 37 °C for 48 hours.

B9.3 Reading of results

Examine the membrane filter for colonies of *Pseudomonas aeruginosa* demonstrating pyocyanin production (green colouration). Colonies may also be blue-green, greenish brown or brown in colour. Also, examine the filter under the UV lamp and count all fluorescent colonies. These colonies, which may or may not be pigmented, should also be considered as presumptive *Pseudomonas aeruginosa*.

B9.4 Confirmation tests

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of pigmented and/or fluorescent colonies. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present. At least ten colonies should be sub-cultured if more than ten are present.

B9.4.1 Milk agar with cetyl trimethylammonium bromide

Subculture each pigmented and/or fluorescent colony to be tested to milk agar with cetyl trimethylammonium bromide and spread so as to obtain single colonies. Incubate at 37 °C for 24 hours. Colonies which are 2 - 4 mm in diameter and show typical pigment production and possess a “halo of clearing” around the colony where the casein has been hydrolysed are recorded as confirmed *Pseudomonas aeruginosa*. Positive (*Pseudomonas aeruginosa*) and negative (for example *Pseudomonas diminuta*) control cultures should be included with each batch of confirmatory tests.

Some strains of *Pseudomonas aeruginosa* may fail to produce pigment on isolation on *Pseudomonas* agar or milk agar with cetyl trimethylammonium bromide. In such cases, exposure to daylight at room temperature for a short time before examination may enhance pigmentation. Where pigment is still not produced, and the presence of *Pseudomonas aeruginosa* are suspected, subculture colonies to a fresh Petri dish of milk agar with cetyl trimethylammonium bromide, and incubate at 37 °C for 24 hours. This provides a pure culture that can then be confirmed using commercially available test-kits, following appropriate performance verification in the laboratory.

B10 Calculations

B10.1 Presumptive *Pseudomonas aeruginosa*

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

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

B10.2 *Confirmed Pseudomonas aeruginosa*

The number of confirmed *Pseudomonas aeruginosa* colonies is calculated by multiplying the number of presumptive *Pseudomonas aeruginosa* colonies by the proportion of isolates that hydrolysed casein.

B11 Expression of results

Counts for presumptive and confirmed *Pseudomonas aeruginosa* are expressed in colony forming units per volume of sample. For drinking water the volume is, typically, 100 ml.

B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Pseudomonas aeruginosa*) and non-target bacteria (for example *Escherichia coli*). Petri dishes should be incubated for 48 hours at 37 °C. Further details are given elsewhere⁽²⁾ in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
4. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. *Journal of Clinical Pathology*, Brown, M. W. R. & Foster, J. H. S., 1970, **23**, 172-177.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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