ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2010) - Part 8 -The isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains two methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration and updates and replaces the earlier version published in 2002. Included in the annex of this document are details of confirmation techniques for *Pseudomonas aeruginosa* based on the use of milk agar supplemented with cetyl trimethylammonium bromide, and the use of Mueller Hinton agar with discs impregnated with 1:10 phenanthroline.

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

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

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 2 - Practices and procedures for sampling (currently undergoing revision)
Part 3 - Practices and procedures for laboratories (currently undergoing revision)
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

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

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

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

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

The Microbiology of Drinking Water (2010)
Part 5 - 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 8 - The isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration

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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.

Performance of methods

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

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

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

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency.

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

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

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

Dr D Westwood
Secretary
February 2010

Warning to users

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

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

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

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

A 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 evidence to suggest that some 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 when incubated at 30 °C for 21 ± 3 hours. 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 on 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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) 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. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

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
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 °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 °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 °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
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 °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 °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.

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 reused. 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.

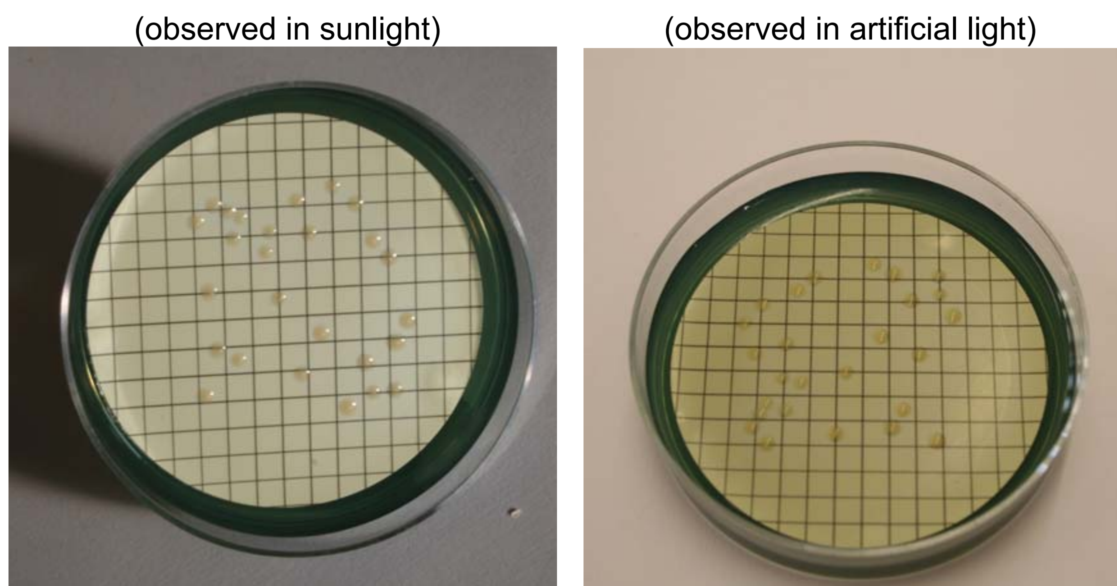
The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours. Incubate the Petri dishes at 30 °C for 20 ± 3 hours.

A9.3 *Reading of results*

After incubation, examine the ampicillin dextrin agar membrane 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, see Figure A1. Colonies that are completely blue or white, or are translucent in appearance should not be counted. Some species of *Vibrio* will grow on ampicillin dextrin agar producing yellow colonies.

Figure A1 Typical colonies of *Aeromonas* on ampicillin dextrin agar

(observed in sunlight) (observed in artificial light)



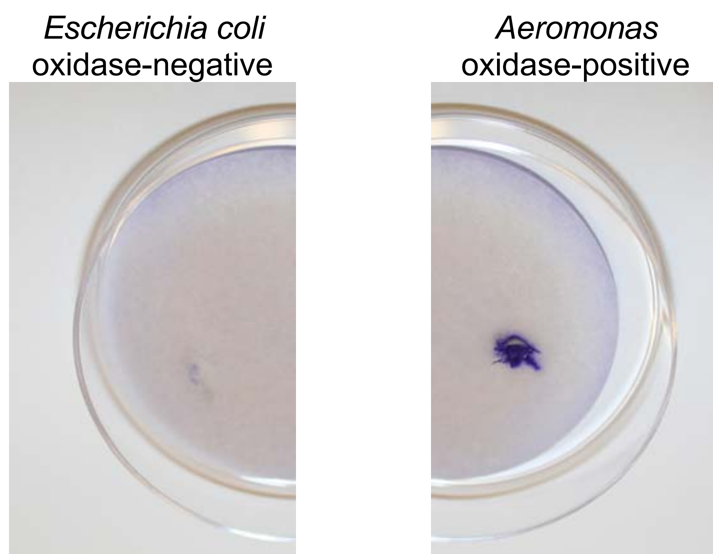
A9.4 *Confirmation tests*

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*

Sub-culture target colonies from ampicillin dextrin agar 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. See Figure A2.

Figure A2 Oxidase test



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, sub-culture 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 up to 48 hours. Alternatively, incubate one tube (without mineral oil) aerobically, and another tube (again, without mineral oil) in an anaerobe jar, both at 30 °C for up to 48 hours. Tubes can be examined after 20 - 24 hours and any positive reaction recorded. See Figure A3.

At the same time inoculate pairs of tubes with organisms known to give a positive fermentation reaction (for example *E. coli,* 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 indicated by a change in colour from green to yellow. An oxidative reaction should show a colour change (from green to yellow) due to acid production in the tube incubated aerobically. There should be no acid production (and hence no colour change) in the tube covered with the mineral oil or the tube incubated anaerobically.

Figure A3 Typical *Hugh and Leifson's O/F test reactions*

a) incubated aerobically for oxidative reaction

E. coli

(oxidative reaction- positive)

Aeromonas

(oxidative reaction- positive)

Pseudomonas

(oxidative reaction- positive)
(weak reaction)

b) incubated in the presence of mineral oil for fermentative reaction

E. coli

(fermentation reaction- positive)

Aeromonas

(fermentation reaction- positive)

Pseudomonas

(fermentation reaction- negative)

c) incubated anaerobically for fermentative reaction

E. coli

(fermentation reaction- positive)

Aeromonas

(fermentation reaction- positive)

Pseudomonas

(fermentation reaction- negative)

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 º 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 (i.e. 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 (i.e. positive sensitivity) should be considered indicative of *Vibrio* species. See Figure A4.

Figure A4 Typical 0129 vibriostatic test reactions

Aeromonas hydrophila

Vibrio parahaemolyticus

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
Aeromonas hydrophila	+	+
Aeromonas caviae	-	+
Aeromonas sobria	+	-
Unidentified *Aeromonas*	-	-

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:

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

Where DF is dilution factor, if appropriate.

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 as appropriate. 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*, Environment Agency, in this series.

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

3. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria, R Hugh and E Leifson, *Journal of Bacteriology*, 1953, **66**, 24-26.

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

5. Prevention of toxicity of metal ions to *Aeromonas* and other bacteria in drinking-water samples using nitrilotriaceticacid (NTA) instead of ethylenediaminetetraaceticacid (EDTA), F Schets and G J Medema, *Letters in Applied Microbiology*, 1993, **16**, 75-76.

6. Ampicillin-dextrin agar medium for the enumeration of *Aeromonas* species in water by membrane filtration, A H Havelaar, M During and J F M Versteegh, *Journal of Applied Bacteriology*, 1987, **62**, 279-287.

7. The presence of *Aeromonas* in drinking water supplies in the Netherlands, A H Havelaar, J F M Versteegh and M During, *Zentralblatt für Hygiene*, 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 all drinking waters. The organisms are 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, hydrolyse casein and are resistant to 1:10 phenanthroline (i.e. continue to grow in its presence).

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 when incubated at 37 °C for 48 hours. Confirmation of isolates is by sub-culture to milk agar supplemented with cetyl trimethylammonium bromide (CMA) to demonstrate hydrolysis of casein, or by sub-culture to Mueller Hinton agar (MHA) followed by addition of discs impregnated with 1:10 phenanthroline.

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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent).

B7.2 Incubator capable of maintaining a temperature of 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. Commercial formulations should be used and stored according to manufacturer’s instructions. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

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
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
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
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.

B8.2.3 *Mueller Hinton Agar*[5]

Beef, dehydrated infusion from	300 g
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Water	1000 ml

Suspend the ingredients in the water and dissolve by heating and stirring. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Cool the solution and dispense suitable volumes into Petri dishes. The final pH of the medium should be 7.3 ± 0.2. Sterile media may be stored for up to one month at a temperature of 5 ± 3 °C if protected from dehydration.

B8.2.4 *1:10 Phenanthroline discs*[5]

These are small discs, 9-10 mm in diameter, containing 80 µg 1:10 phenanthroline. Not all commercially available discs may be suitable. If commercial impregnated discs are used they should be stored according to manufacturer's instructions.

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 quarter-strength 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.

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 reused. 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 incubated membrane filter after 24 hours and again after 48 hours for colonies of *Pseudomonas aeruginosa* demonstrating pyocyanin production (green colouration). See Figure B1. The number of colonies at 24 hours may need to be noted as growth between 24 and 48 hours may be such that colonial growth results in the merging of colonies, and the number of colonies at 48 hours may be less than the number of colonies at 24 hours. Colonies may also be blue-green, greenish brown or brown in colour. Also, examine the filter under the UV lamp (see Figure B2) and count all fluorescent colonies. These colonies, which may or may not be pigmented, should also be considered as presumptive *Pseudomonas aeruginosa*.

Figure B1 *Pseudomonas aeruginosa* growing on *Pseudomonas* agar

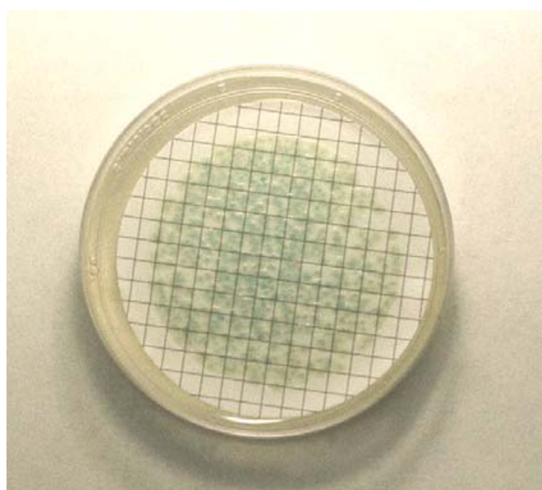
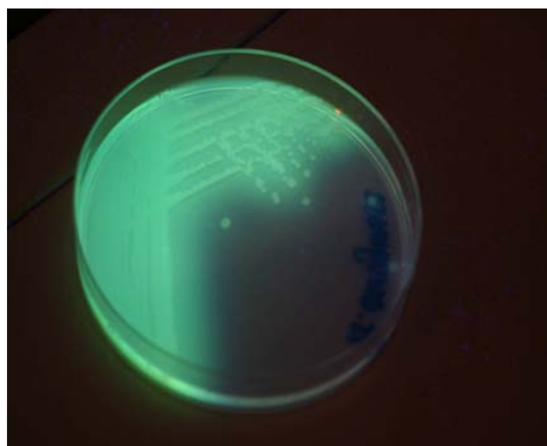


Figure B2 ***Pseudomonas aeruginosa* growing on *Pseudomonas* agar**

As seen under UV light	Diffusable green pigment
	

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.

Typical colonies that are green or blue-green in colour and are also oxidase-positive may not require further confirmation. See flowchart B1.

B9.4.1 *Oxidase test*

Sub-culture target colonies from *Pseudomonas* agar 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 and *Pseudomonas aeruginosa* are oxidase-positive. See Figure B3.

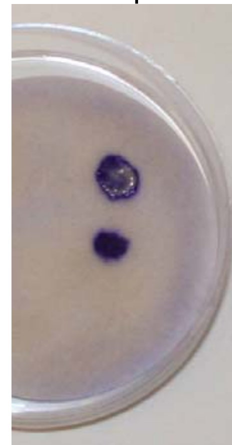
Commercial test kits for oxidase testing are available and should be used in accordance with the manufacturer's instructions and following appropriate performance verification at the laboratory.

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*).

Figure B3 Oxidase test

Escherichia coli
oxidase-negative

Pseudomonas aeruginosa
oxidase-positive

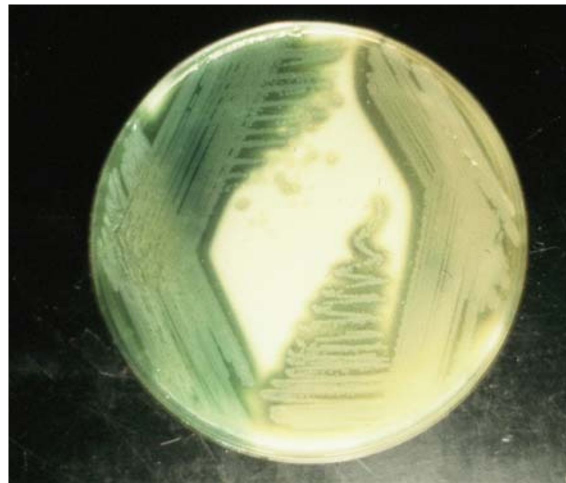


B9.4.2 *Milk agar with cetyl trimethylammonium bromide*

Sub-culture each pigmented and/or fluorescent colony to be tested from *Pseudomonas* agar to milk agar with cetyl trimethylammonium bromide (CMA) and spread so as to obtain single colonies. Incubate at 37 °C overnight. Colonies which are 2 - 4 mm in diameter and show typical pigment production and possess an "area of clearing" around the colony where the casein has been hydrolysed are recorded as confirmed *Pseudomonas aeruginosa.* See Figure B4. Positive (for example *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, sub-culture colonies to a fresh Petri dish of milk agar with cetyl trimethylammonium bromide, and incubate at 37 °C overnight. This provides a pure culture that can then be confirmed using commercially available test-kits, following appropriate performance verification in the laboratory.

Figure B4 Casein hydrolysis on CMA

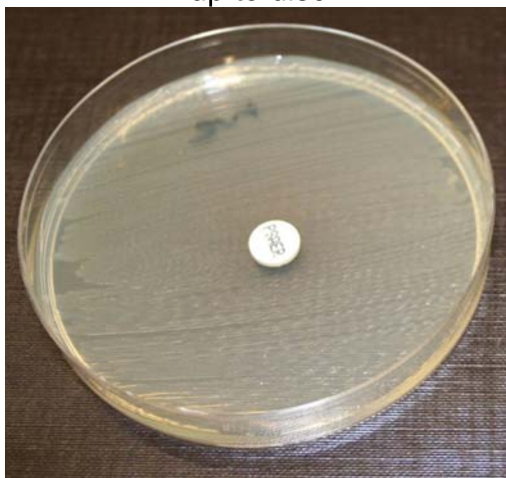


B9.4.3 MHA and 1:10 phenanthroline test

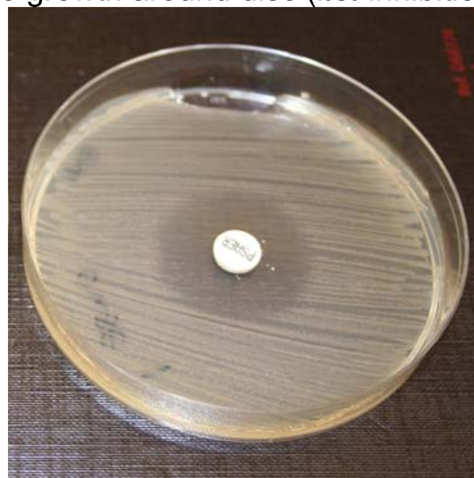
Alternatively, using a sterile loop, sub-culture each pigmented and/or fluorescent colony to be tested from *Pseudomonas* agar to a Petri dish containing dry Mueller Hinton agar (MHA). Smear the agar surface to generate an area of confluent growth. Rotate the dish 90 degrees and smear again. Using flame-sterilised forceps, asceptically transfer a disc impregnated with 1:10 phenanthroline onto the centre of the agar. The Petri dish is incubated at 37 °C overnight. Growth up to the disc exhibiting typical pigmentation or fluorescence is reported as confirmed *Pseudomonas aeruginosa*. See Figure B5. Isolates displaying zones of inhibition in proximity to the disc are not regarded as *Pseudomonas aeruginosa*. See Appendix 1 for comparison data.

Figure B5 Typical example of MHA with disc impregnated with 1:10 phenanthroline

Pseudomonas aeruginosa showing growth up to disc



Pseudomonas fluorescens showing zone of no growth around disc (i.e. inhibition)



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:

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

Where DF is dilution factor, if appropriate.

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 on CMA or are resistant to 1:10 phenanthroline on MHA.

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 as appropriate. 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*, Environment Agency, in this series.

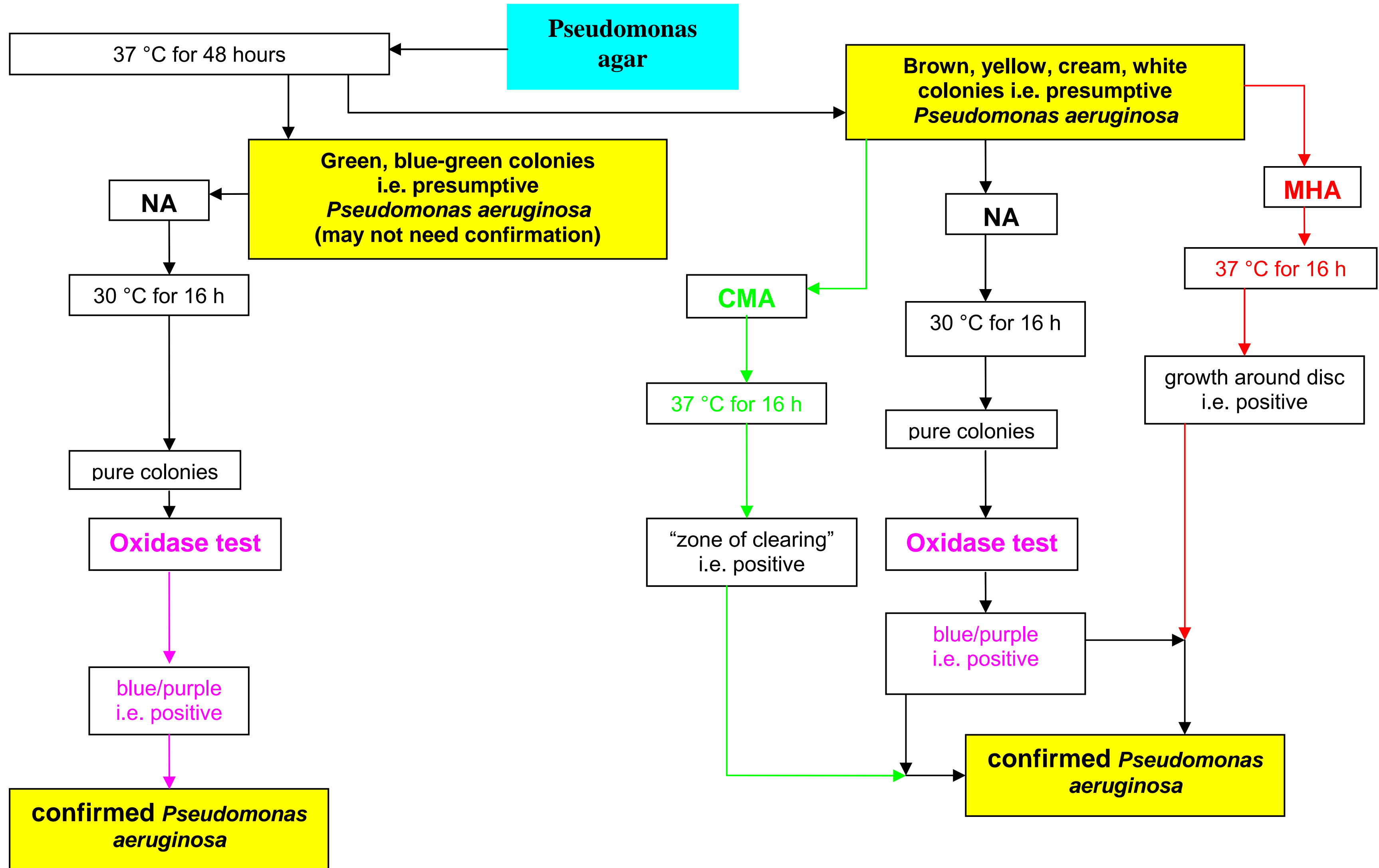
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency, in this series. (currently undergoing revision.)

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

4. A simple diagnostic milk medium for *Pseudomonas aeruginosa*, M W R Brown and J H S Foster, *Journal of Clinical Pathology*, 1970, **23**, 172-177.

5. Identification of *Pseudomonas aeruginosa* with "*Ps. aeruginosa* Screen" diagnostic tablets, N Pringler and J B Casals, Proceedings of the 5th European Congress of Clinical Microbiology and Infectious Diseases, 1991, Oslo, Norway.

Flow chart B1 **Confirmation of colonies of presumptive *Pseudomonas aeruginosa* on colonies obtained on Pseudomonas agar incubated at 37 °** (see section B9.4)



Appendix 1 Confirmation of *Pseudomonas aeruginosa* by testing for resistance to 1:10 phenanthroline compared to the procedure described previously in MoDW (2002) - Part 8

1 Introduction

The conventional test described previously in this series[1] for the confirmation of *Pseudomonas aeruginosa* in water samples involves sub-culture from Pseudomonas agar to milk agar supplemented with cetyl trimethylammonium bromide (CMA) for demonstrating the hydrolysis of casein. *Pseudomonas aeruginosa* hydrolyses casein (indicated by a "zone of clearance" around colonies) with typical pigmentation of colonies.

An alternative confirmation procedure has been proposed by Working Group 2 (WG2) members of the Standing Committee of Analysts (SCA) for the confirmation of *Pseudomonas aeruginosa* using Mueller Hinton agar (MHA) and discs (9 mm in diameter) impregnated with 80 µg of 1:10 phenanthroline. *Pseudomonas aeruginosa* are resistant to 1:10 phenanthroline. Other species of *Pseudomonas* are sensitive to 1:10 phenanthroline which results in zones of no growth around the discs[2]. It has been reported[2] that 92 of 100 strains of *Pseudomonas aeruginosa* showed no zones of inhibition, six strains exhibited zones of inhibition of less than 12 mm diameter (i.e. not more than 1.5 mm from the disc edge) whilst two strains exhibited zones of 15 mm and 18 mm diameter respectively (i.e. 3 mm and 4.5 mm from disc edge respectively). In contrast, 87 strains of other species of *Pseudomonas* (including four strains of *Pseudomonas fluorescens*, eight strains of *Pseudomonas putida* and 21 strains of *Burkholderia cepacia*) showed zones of inhibition of between 18 - 37 mm diameter. Additionally, 121 other strains of non-fermenting Gram-negative bacilli and Enterobacteriaceae showed zones of inhibition of 19 - 35 mm diameter. It has been proposed[3] that discs impregnated with 25 µg of 1:10 phenanthroline in combination with 25 µg of the bacteriostatic agent C-390 (9-chloro-9-[4-(diethylamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride) may be used for the confirmation of *Pseudomonas aeruginosa*. Sensitivity and specificity values for the confirmation of *Pseudomonas aeruginosa* based on resistance to the bacteriostatic agents and pyocyanin production are reported as 99 % and 100 % respectively.

This new study was conducted under the auspices of SCA WG2 to assess whether the alternative procedures could be regarded as equivalent to, or better than, the CMA casein hydrolysis test for the confirmation of *Pseudomonas aeruginosa*.

2 Materials and Methods

Nine laboratories participated in the study. Each laboratory analysed samples from a variety of water types (including chlorinated and unchlorinated waters, and treated and waste waters).

2.1 Primary isolation of presumptive *Pseudomonas aeruginosa*

Presumptive *Pseudomonas aeruginosa* were isolated using membrane filtration and incubation on Pseudomonas agar supplemented with nalidixic acid and cetyl trimethylammonium bromide (Oxoid Pseudomonas Agar base, CM0559B, and supplement, SR0102E, or equivalent) for 48 hours at 37.0 ± 1.0 °C according to procedures described in The Microbiology of Drinking Water - Part 8[1]. The sample source type was recorded, for example drinking water, swimming pool, etc. Appropriate sample volumes (for example 100 ml, 10 ml or 1 ml) based on previous experience of the

water source were filtered such that, as far as practicable, the membrane filters contained between 10 - 30 colonies, that could be used for subsequent confirmation. Colonies were counted in accordance with procedures described previously[1]. Colonies which demonstrated pyocyanin production (i.e. green colouration) or were primarily blue-green, greenish brown or brown in colour were counted. Colonies were also examined under UV light (approximately 350 nm) and fluorescent colonies were counted. Presumptive counts were recorded together with respective colony colouration and any fluorescence observed.

2.2 Confirmation of isolates

Up to 10 typical presumptive colonies from each membrane filter were selected and tested for oxidase reaction before confirmation tests were carried out. Since *Pseudomonas aeruginosa* are oxidase-positive, oxidase-negative colonies were not subjected to further confirmation tests. A representative number of presumptive *Pseudomonas aeruginosa* colonies were then streaked onto Mueller Hinton agar (MHA) (Oxoid Muller Hinton agar, CM0337B, or equivalent) and also on to milk agar supplemented with cetyl trimethylammonium bromide (CMA). For the Petri dishes containing MHA, streaking was conducted so as to generate an area of confluent growth. A disc impregnated with 1:10 phenanthroline (Rosco Pseudomonas tablets, ref 593-11, Bioconnections, Wetherby) was then placed onto the MHA streaks. Both the MHA and CMA Petri dishes were incubated overnight at 37.0 ± 1.0 °C. Growth with typical pigment production and a “zone of clearing” on the CMA was recorded as confirmed *Pseudomonas aeruginosa*, as was growth up to the phenanthroline-impregnated disc on MHA, with pigmentation and/or fluorescence. Isolates displaying no “zone of clearing” on the CMA, or zone of inhibition around the impregnated disc on MHA, were not regarded as confirmed *Pseudomonas aeruginosa*. If a discrepancy between the results of the two confirmation tests were reported the isolate was identified using other tests such as the API 20NE (BioMerieux) test or similar identification system suitable for *Pseudomonas aeruginosa*.

2.3 Quality control

All the media used for the trial was prepared and quality controlled in accordance with procedures described in The Microbiology of Water 2002 - Part 3[4]. A known strain of *Pseudomonas aeruginosa* (for example NCTC 10662) was included as a positive control for the whole procedure. It was, however, only necessary to filter one sample containing the control organism and to sub-culture one colony for confirmation with the other sub-cultures. It was not necessary to enumerate counts from the membrane filter but merely to assure that the control organisms were satisfactory. Reference lenticulated *Pseudomonas aeruginosa* could also be used as positive control organisms. A negative control strain, such as *Pseudomonas diminuta* (for example NCTC 8545) was also used.

3 Results and Discussion

Nine sets of data were received. In total, 218 samples were analysed from a variety of water types and from these waters, 1523 isolates were subjected to confirmatory testing. The number of samples and results are summarised in Table 1. The number of samples with respect to the various water types analysed are summarised in Table 2.

The data were analysed to assess whether there was any significant difference between the two sub-culture procedures for the confirmation of *Pseudomonas aeruginosa*, or whether indeed there was a need for confirmatory testing of typical colonies.

From Table 1, the number of presumptive *Pseudomonas aeruginosa* (i.e. 1233, showing fluorescence under UV illumination and being oxidase-positive) was 81 %.

Laboratories were requested to count those colonies demonstrating typical pyocyanin production, i.e. green, blue-green, greenish brown or brown colour colonies. A proportion of cream and white colonies were also detected and sub-cultured for confirmation together with several yellow colonies. The confirmation rates for all colour types are summarised in Table 3. Green and blue-green colonies showed the highest confirmation rates as expected based on typical pyocyanin production. Very little difference is seen in confirmation rates between the two test methods for green and blue-green colony types (see Table 3) with 90.8 % of green colonies confirmed as *Pseudomonas aeruginosa* on CMA, and 90.6 % confirmed by the phenanthroline test, and 100 % confirmation rates shown by both tests for blue-green colonies. It might be inferred from these results that green and blue-green colonies, generally, do not require confirmation.

For the other colonies (i.e. brown, cream/white and yellow colonies) approximately 40 % of presumptive isolates confirmed as *Pseudomonas aeruginosa* by both confirmation tests, except for yellow colonies with 1:10 phenanthroline where only 24 % confirmed. However, the number of colonies tested is relatively small, see Table 3.

Overall, as the following table shows, 96.3% agreement (i.e. 1071 positive results by both tests and 396 negative results by both tests, i.e. 1467 results from 1523 colonies tested) is shown between the two procedures. Individual laboratory results are shown in Table 5. Out of all the laboratories only one laboratory, laboratory 4, showed a significant difference in the percent agreement values.

		CMA			
		+	-		
Phenanthroline test	+	**1071**	23	1094	
	-	33	**396**	429	
		1104	419	1523	agreement = 96.3%

From Table 1 the total average confirmation rate for CMA was 72.5%, and the total average confirmation rate for the phenanthroline test was 71.8%. It can also be seen from Table 1 that whilst most laboratories showed no significant differences between the confirmation rates for the two media, i.e. CMA and MHA with 1:10 phenanthroline, laboratory 5 showed significantly higher confirmation rates (approximately 100 %) compared with the other laboratories (approximately 55 - 75 %) . In addition, laboratory 4 showed a lower confirmation rate for the 1:10 phenanthroline test. Laboratory 4 analysed only 14 samples (42 isolates), mainly from a warm spring water source. This is also reflected in general low confirmation rates shown in Table 2 for spring waters. From Table 2 it can also be seen that there is no significant difference in the confirmation rates between the two tests shown for isolates taken from other sources of water.

Further (identification) tests were carried out where a difference was observed between the two confirmation tests. Table 4 shows a summary of these data. Of those isolates where there was reliable identification, CMA showed 20 false-positive and 12 false-negative results. However, the 1:10 phenanthroline test showed only 2 false-positive and 1 false-negative reactions. The isolates producing false-positive “zones of clearing” on CMA were identified as *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aeromonas hydrophila* and *Alcaligenes faecalis*. The two isolates showing false-positive resistance to phenanthroline were identified as *Burkholderia cepacia*. Thus, although the overall

confirmation rate was slightly higher for CMA, not all of these isolates were identified as *Pseudomonas aeruginosa*, compared to those for the phenanthroline test result.

4 Conclusions

This study compared two methods for the confirmation of presumptive *Pseudomonas aeruginosa* isolated on Pseudomonas agar supplemented with nalidixic acid and cetyl trimethylammonium bromide from a variety of water types. The principal conclusions are:

(i) A confirmation agreement rate of 96.3% is showed between the two confirmation methods. However, the 1:10 phenanthroline test showed fewer false-positive and false-negative reactions, compared with milk agar supplemented with cetyl trimethylammonium bromide.

(ii) Where green or blue-green colonies are isolated on Pseudomonas agar supplemented with nalidixic acid and cetyl trimethylammonium bromide, and these colonies are oxidase-positive and show fluorescence, then confirmatory tests may not be required.

There is general opinion that the phenanthroline test is easier to perform than the CMA test and that the MHA medium is easier to prepare than the CMA medium.

For a range of water samples involving over 1500 isolates, the 1:10 phenanthroline resistance confirmation procedure has been shown to produce results that are similar to, or better than, corresponding results using the CMA test.

5 Acknowledgements

The SCA is indebted to the managers and analysts of the following laboratories for their participation in this study-

CREH *Analytical* (Leeds)
Northumbrian Water Scientific Services (Horsley)
Scottish Water (Edinburgh)
Severn Trent Laboratories (Coventry)
Severn Trent Water (Nottingham)
Severn Trent Water (Shrewsbury)
South East Water (Frimley, Surrey)
Southern Water (Winchester)
Wessex Water (Bath)

The study was organised and managed by Phil Holmes (Severn Trent Water), and thanks are expressed to David Sartory (SWM consulting) for valuable comments and assistance.

6 References

1. Standing Committee of Analysts, 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*, Environment Agency, in this series.

2. Identification of *Pseudomonas aeruginosa* with "*Ps. aeruginosa* Screen" diagnostic tablets, N Pringler and J B Casals, Proceedings of the 5th European Congress of Clinical Microbiology and Infectious Diseases, 1991, Oslo, Norway.

3. Identification of *Pseudomonas aeruginosa* by using a disk of phenanthroline and C390 and by cell agglutination testing with monoclonal antibodie, S Fujita, A Tonohata, T Matsuoka, N Okado and T Hashimoto, *Journal of Clinical Microbiology*, 1992, **30**, 2728-2729.

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

Table 1 **Summary of *Pseudomonas aeruginosa* confirmation results by laboratory for casein hydrolysis on milk agar supplemented with cetyl trimethylammonium bromide (CMA) and resistance to 1:10 phenanthroline**

		Pseudomonas agar			CMA		MHA - 1:10 phenanthroline test	
Laboratory number	Samples analysed	Number of colonies used for confirmation	Number of typical colonies	Presumptive *Pseudomonas aeruginosa* (%)	Number of positive colonies	Confirmed as *Pseudomonas aeruginosa* (%)	Number of positive colonies	Confirmed as *Pseudomonas aeruginosa* (%)
1	46	104	94	90.4	68	65.4	67	64.4
2	30	302	133	44.0	218	72.2	224	74.2
3	11	98	82	89.1	72	73.5	72	73.5
4	14	42	33	78.6	28	66.7	18	42.9
5	30	298	298	100	297	99.7	298	100
6	31	253	191	75.5	161	63.6	156	61.7
7	17	172	158	91.9	97	56.4	97	56.4
8	27	196	186	91.8	127	64.8	122	62.2
9	12	58	58	100	36	62.1	40	69.0
	218	**1523**	**1233**	**81.0**	**1104**	**72.5**	**1094**	**71.8**

Table 2 **Summary of *Pseudomonas aeruginosa* confirmation results by water type for casein hydrolysis on milk agar supplemented with cetyl trimethylammonium bromide (CMA) and resistance to 1:10 phenanthroline**

Water type		*Pseudomonas* agar		CMA		MHA - 1:10 phenanthroline test	
	Number of colonies used for confirmation	Number of typical colonies	Presumptive *Pseudomonas aeruginosa* (%)	Number of positive colonies	Confirmed as *Pseudomonas aeruginosa* %	Number of positive colonies	Confirmed as *Pseudomonas aeruginosa* %
Drinking	344	198	67.3	257	74.7	267	78.5
Raw/River	433	391	90.3	337	77.8	327	75.5
Swimming pool/Spa	231	202	87.4	146	63.2	148	64.1
Sewage	316	256	81.0	235	74.4	240	75.9
Spring	64	24	37.5	23	36.0	11	17.2
Sea	95	81	85.3	66	69.5	66	69.5
Other	40	36	90.0	40	100	35	87.5
	1523	**1233**	**81.0**	**1104**	**72.5**	**1094**	**71.8**

Table 3 Summary of *Pseudomonas aeruginosa* confirmation results by colony colour for casein hydrolysis on milk agar supplemented with cetyl trimethylammonium bromide (CMA) and resistance to 1:10 phenanthroline

		Pseudomonas agar		CMA		MHA - 1:10 phenanthroline test	
Colony colour	Number of colonies used for confirmation	Number of typical colonies	Presumptive *Pseudomonas aeruginosa* (%)	Number of positive colonies	Confirmed as *Pseudomonas aeruginosa* (%)	Number of positive colonies	Confirmed as *Pseudomonasa aeruginosa* (%)
Green	946	945	99.9	859	90.8	857	90.6
Blue-green	10	10	100	10	100	10	100
Brown	380	164	43.2	162	42.6	156	41.0
Cream/White	162	99	61.1	62	38.3	65	40.1
Yellow	25	15	60.0	11	44.0	6	24.0
	1523	**1233**	**81.0**	**1104**	**72.5**	**1094**	**71.8**

Table 4 **Identification of isolates which differed in reactions for casein hydrolysis in milk agar supplemented with cetyl trimethylammonium bromide (CMA) and resistance to MHA - 1:10 phenanthroline**

Colony colour	Number of colonies	CMA		MHA - 1:10 phenanthroline test		Identification	Interpretation
		+	-	+	-		
Brown	1	✓			✓	No ID, negative fluorescence	Probably not *Pseudomonas aeruginosa*
Brown	1	✓			✓	*Pseudomonas putida*	CMA false-positive
Brown	7		✓	✓		*Pseudomonas. aeruginosa*	CMA false-negative
Green	3		✓	✓		*Pseudomonas aeruginosa*	CMA false-negative
Green	11	✓			✓	6 *Pseudomonas putida*, 5 *Pseudomonas fluorescens*	CMA false-positive
Green	2	✓			✓	*Pseudomonas. fluorescens*	CMA false-positive
Green	1	✓			✓	*Pseudomonas aeruginosa*	Phenanthroline false-negative
Green	1		✓	✓		*Pseudomonas aeruginosa*	CMA false-negative
Green	1		✓	✓		*Pseudomonas aeruginosa*	CMA false-negative
Brown	4	✓			✓	*Aeromonas hydrophila*	CMA false-positive
Brown	2		✓	✓ weak		*Burkholderia cepecia*	Phenanthroline false-positive
Brown	2	✓weak			✓	*Alcaligenes faecalis*	CMA false-positive
Brown	1	✓ weak			✓	No ID, negative fluorescence	Probably not *Pseudomonas aeruginosa*
Brown	5	✓			✓	No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*
Green	3		✓	✓		No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*
Cream	2		✓	✓		No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*
Yellow	1	✓			✓	No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*
Yellow	4	✓			✓	No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*
Green	4		✓	✓		No ID, positive fluorescence	Possibly *Pseudomonas aeruginosa*

From data with reliable identification		
	CMA	false-positive = 20
	CMA	false-negative = 12
	MHA - 1:10 phenanthroline test	false-positive = 2
	MHA - 1:10 phenanthroline test	false-negative = 1

Table 5 **Confirmation results by the casein hydrolysis test (CMA) and resistance to 1:10 phenanthroline from individual laboratories**

Laboratory 1

		CMA +	CMA -		
Phenanthroline test	+	**67**	0	67	
	-	1	**36**	37	
		68	36	104	agreement = 99.0 %

Laboratory 2

		CMA +	CMA -		
Phenanthroline test	+	**217**	7	224	
	-	1	**77**	78	
		218	84	302	agreement = 97.4 %

Laboratory 3

		CMA +	CMA -		
Phenanthroline test	+	**72**	0	72	
	-	0	**26**	26	
		72	26	98	agreement = 100 %

Laboratory 4

		CMA +	CMA -		
Phenanthroline test	+	**14**	4	18	
	-	14	**10**	24	
		28	14	42	agreement = 57.1 %

Laboratory 5

		CMA +	CMA -		
Phenanthroline test	+	**297**	1	298	
	-	0	**0**	0	
		297	1	298	agreement = 99.7 %

Laboratory 6

		CMA +	CMA -		
Phenanthroline test	+	**154**	2	156	
	-	7	**90**	97	
		161	92	253	agreement = 96.4 %

Laboratory 7

		CMA +	CMA -		
Phenanthroline test	+	**97**	0	97	
	-	0	**75**	0	
		97	0	172	agreement = 100 %

Laboratory 8

		CMA +	CMA -		
Phenanthroline test	+	**117**	5	122	
	-	10	**64**	74	
		127	69	196	agreement = 94.4 %

Laboratory 9

		CMA +	CMA -		
Phenanthroline test	+	**36**	4	40	
	-	0	**18**	18	
		36	22	58	agreement = 93.1 %

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.

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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.

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