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Water quality — Detection and enumeration of *Pseudomonas aeruginosa* — Method by membrane filtration

Qualité de l'eau — Recherche et dénombrement de Pseudomonas aeruginosa *— Méthode par filtration sur membrane*

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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16266 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This International Standard is the equivalent of EN 12780:2002.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of man that is capable of growth in water at very low nutrient concentrations. At source and during marketing, a natural mineral water or a spring water is to be free from *Pseudomonas aeruginosa* in any 250 ml sample examined (see, e.g. Council Directive 80/777/EEC[1] and Council Directive 96/70/EC[2]). Other bottled waters offered for sale are also to be free of *Pseudomonas aeruginosa* in any 250 ml sample (see, e.g. Council Directive 98/83/EC[3]). Other waters, including pool waters and water for human consumption, may sometimes be tested for *Pseudomonas aeruginosa* for reasons of public health. In these cases, it is typical to examine 100 ml volumes.

Water quality — Detection and enumeration of *Pseudomonas aeruginosa* — Method by membrane filtration

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the isolation and enumeration of *Pseudomonas aeruginosa* in samples of bottled water by a membrane filtration technique. This method can also be applied to other types of water with a low background flora, for example, pool waters and waters intended for human consumption.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

ISO 5667-2[1)], *Water quality — Sampling — Part 2: Guidance on sampling techniques*

ISO 5667-3, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of water samples*

ISO 6887-1, *Microbiology of food and feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

ISO 19458[2)], *Water quality — Sampling for microbiological analysis*

1) ISO 5667-1 and ISO 5667-2 are currently undergoing joint revision, which will be published as ISO 5667-1.

2) To be published.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
Pseudomonas aeruginosa
micro-organisms that grow on selective media containing cetrimide and produce pyocyanin, or micro-organisms that grow on selective media containing cetrimide, are oxidase positive, fluoresce under UV radiation (360 ± 20) nm, and are able to produce ammonia from acetamide

4 Principle

4.1 Filtration

A measured volume of the water sample, or a dilution of the sample, is filtered through a membrane filter of 0,45 µm. The membrane filter is placed on the selective medium and incubated under the conditions specified for the medium.

4.2 Enumeration

The numbers of presumptive *Pseudomonas aeruginosa* are obtained by counting the number of characteristic colonies on the membrane filter after incubation. Pyocyanin-producing colonies are considered as confirmed *Pseudomonas aeruginosa* but other fluorescing or reddish brown colonies require confirmation.

4.3 Confirmation

Subcultures of colonies requiring confirmation are made from the membrane filter onto plates of nutrient agar (but see Annex B). After incubation, cultures that were not initially fluorescent are tested for the oxidase reaction, and oxidase-positive cultures are tested for the production of fluorescein and the ability to produce ammonia from acetamide. Cultures that were fluorescent initially are tested for the ability to produce ammonia from acetamide.

5 Diluents, culture media and reagents

Use reagents of analytical reagent quality in the preparation of culture media and diluents, unless otherwise specified. Prepare the medium as follows and add the selective agents as supplements at the given concentrations or use commercially available media and reagents prepared according to the manufacturer's instructions. Prepare media and reagents using water grade 3 as specified in ISO 3696, or water of equivalent purity and free from substances which might inhibit growth under the conditions of the test.

5.1 Culture medium

Use the following medium for the determination of *Pseudomonas aeruginosa*.

5.1.1 *Pseudomonas* agar base/CN-agar

5.1.1.1 Composition

Gelatin peptone	16,0 g
Casein hydrolysate	10,0 g
Potassium sulfate (anhydrous) (K_2SO_4)	10,0 g

Magnesium chloride (anhydrous) ($MgCl_2$)	1,4 g
Glycerol	10 ml
Agar	11,0 g to 18,0 g
Water (distilled or equivalent)	1 000 ml

NOTE The amount of agar required depends on the gel strength. Follow the manufacturer's instructions for the agar used.

CN supplement

Hexadecyltrimethyl ammonium bromide (cetrimide)	0,2 g
Nalidixic acid	0,015 g

5.1.1.2 Preparation

Suspend the peptone, casein hydrolysate, potassium sulfate, magnesium chloride and agar in 1 000 ml of distilled water (or equivalent). Add 10 ml of glycerol. Heat to boiling in order to dissolve completely and sterilize by autoclaving at (121 ± 3) °C for 15 min. Allow the medium to cool to (45 to 50) °C. Add the CN supplement rehydrated in 2 ml of sterile distilled water, mix well and add to the sterile molten basal medium. Mix well and pour into sterile Petri dishes to give a depth of at least 5 mm of agar. The final pH of the solidified medium should correspond to 7,1 ± 0,2 at 25 °C. Store prepared plates in the dark protected from desiccation at (5 ± 3) °C and use within 1 month. Do not keep the agar molten for more than 4 h. Do not remelt the medium.

5.2 Confirmatory media and reagents

5.2.1 King's B medium

5.2.1.1 Composition

Peptone	20,0 g
Glycerol	10 ml
Di-potassium hydrogen phosphate (K_2HPO_4)	1,5 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	1,5 g
Agar	15,0 g
Water (distilled or equivalent)	1 000 ml

5.2.1.2 Preparation

Dissolve the ingredients in the water by heating. Cool down to (45 to 50) °C and adjust the pH corresponding to 7,2 ± 0,2 at 25 °C, using either hydrochloric acid or sodium hydroxide. Dispense the medium in 5 ml aliquots into culture tubes which are capped and autoclaved at (121 ± 3) °C for 15 min. Allow the tubes to cool and solidify in slants.

Store in the dark at (5 ± 3) °C and use within 3 months.

5.2.2 Acetamide broth

5.2.2.1 Composition

Solution A

Potassium di-hydrogenphosphate (KH_2PO_4)	1,0 g
Magnesium sulfate (anhydrous) ($MgSO_4$)	0,2 g
Acetamide	2,0 g
Sodium chloride (NaCl)	0,2 g
Water (distilled or equivalent, ammonia free)	900 ml

Dissolve the ingredients in water and then adjust the pH to correspond to 7,0 ± 0,5 at 25 °C with either hydrochloric acid or sodium hydroxide.

CAUTION — Acetamide is carcinogenic and irritant — appropriate precautions shall be taken when weighing out, preparing and discarding the medium.

Solution B

Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0,5 g
Iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	0,05 g
Water	100 ml

5.2.2.2 Preparation

To prepare the acetamide broth, add 1 ml of solution B to 900 ml of a freshly prepared solution A (5.2.2.1). Add water with constant stirring to a total volume of 1 l. Dispense this mixture in 5 ml aliquots to culture tubes which are then capped and sterilized in an autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C and use within 3 months.

5.2.3 Nutrient agar

5.2.3.1 Composition

Peptone	5,0 g
Meat extract	1,0 g
Yeast extract	2,0 g
Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water	1 000 ml

5.2.3.2 Preparation

Dissolve the ingredients in the water by heating. Sterilize by autoclaving at (121 ± 3) °C for 15 min. The pH of the solidified prepared medium should correspond to 7,4 ± 0,2 at 25 °C. Dry the plates to remove excess surface moisture before use. Store prepared plates in the dark protected from desiccation at (5 ± 3) °C and use within 1 month.

5.2.4 Oxidase reagent

5.2.4.1 Composition

Tetramethyl-*p*-phenylenediamine dihydrochloride	0,1 g
Water	10 ml

5.2.4.2 Preparation

Dissolve the tetramethyl-*p*-phenylenediamine dihydrochloride in the water immediately before use and protect from light. This reagent is not stable. Prepare in small amounts freshly before use.

Alternatively, use commercially available oxidase tests.

5.2.5 Nessler reagent

5.2.5.1 Composition

Mercuric chloride ($HgCl_2$)	10 g
Potassium iodide (KI)	7 g
Sodium hydroxide (NaOH)	16 g
Water (ammonia free)	to 100 ml

Dissolve 10 g of $HgCl_2$ and 7 g of KI in a small quantity of water and add this mixture slowly, with stirring, to a cooled solution of 16 g of NaOH dissolved in 50 ml of water. Dilute to 100 ml. Store in rubber-stoppered borosilicate glassware out of sunlight for a maximum of 1 year.

CAUTION — $HgCl_2$ is toxic – avoid ingestion.

6 Apparatus and glassware

Use usual microbiological laboratory equipment.

6.1 Glassware

Sterilize all glassware at (170 ± 5) °C for 1 h in a dry oven or at (121 ± 3) °C for 15 min in an autoclave before use.

6.2 Incubator, capable of being maintained at (36 ± 2) °C.

6.3 Ultra violet lamp, capable of emitting radiation of wavelength (360 ± 20) nm.

6.4 Sterile membrane filters, with nominal pore size of 0,45 µm.

Check filters on a regular basis as specified in ISO 7704.

7 Sampling

Carry out the collection, preservation and handling of samples as specified in ISO 5667-1, ISO 5667-2, ISO 5667-3 and ISO 19458.