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16266:2008

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

ICS 13.060.70

National foreword

This British Standard is the UK implementation of EN ISO 16266:2008. It supersedes BS EN 12780:2002 which is withdrawn.

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Foreword

The text of ISO 16266:2006 has been prepared by Technical Committee ISO/TC 147 “Water quality” of the International Organization for Standardization (ISO) and has been taken over as EN ISO 16266:2008 by Technical Committee CEN/TC 230 “Water analysis” the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2008, and conflicting national standards shall be withdrawn at the latest by August 2008.

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The text of ISO 16266:2006 has been approved by CEN as a EN ISO 16266:2008 without any modification.

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Foreword

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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.

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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*

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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.

8 Procedure

8.1 General

Carry out the membrane filtration technique as specified in ISO 8199, and prepare the dilutions as specified in ISO 6887-1.

8.2 Membrane filtration

Filter volumes of the water sample or portions of the dilution through a sterile cellulose ester membrane filter with a rated pore diameter equivalent to 0,45 µm. As specified in ISO 8199, place each membrane on a Petri dish containing CN agar (5.1) ensuring no air is trapped beneath the membrane.

8.3 Incubation of plates

Incubate the Petri dishes at (36 ± 2) °C for (44 ± 4) h in containers and protect against desiccation.

8.4 Examination of membranes

Examine the membranes for growth after (22 ± 2) h and (44 ± 4) h.

Count all colonies that produce blue/green (pyocyanin) colour as confirmed *Pseudomonas aeruginosa.*

Examine the membrane under UV radiation. Note that prolonged periods under UV illumination should be avoided otherwise the colonies may be killed and fail to grow on the confirmatory media. Count all non-pyocyanin producing colonies that fluoresce as presumptive *Pseudomonas aeruginosa* and confirm their identity using acetamide broth as described below.

Count all other reddish brown pigmented colonies that do not fluoresce as presumptive *Pseudomonas aeruginosa* and confirm their identity using the oxidase test, acetamide broth, and King's B media as described below. The reading after (22 ± 2) h is performed in case of overgrowth and merging of colonies which may occur after (44 ± 4) h. Whichever count is the highest should be used to calculate the number of *Pseudomonas aeruginosa* in Clause 9.

Table 1 summarizes the selection of colonies and confirmation steps.

Table 1 — Steps required for the confirmation of colonies growing on CN agar

Description of colony on CN agar	Ammonia from acetamide	Production of oxidase	Fluorescence on King's B	Confirmed as *Pseudomonas aeruginosa*
Blue/green	NT[a]	NT	NT	Yes
Fluorescent (not blue/green)	+	NT	NT	Yes
Reddish brown	+	+	+	Yes
Other types	NT	NT	NT	No

[a] NT: not tested.

8.5 Confirmation

8.5.1 Nutrient agar

Subculture all, or if impracticable as many as possible (see ISO 8199) of the colonies requiring confirmation from the membrane filter and incubate for (22 ± 2) h at (36 ± 2) °C. Check the subcultures for purity and test those that were initially reddish brown for the oxidase reaction (8.5.2).

8.5.2 Oxidase test

Place 2 to 3 drops of freshly prepared oxidase reagent (5.2.4) onto a filter paper in a Petri dish.

With a platinum (not Ni chrome) wire loop, plastics loop, stick or glass rod, smear some of the growth on the prepared filter paper. Regard the appearance of a deep blue-purple colour within 10 s as a positive reaction. Alternatively, use commercially available oxidase tests following the manufacturers instructions.

8.5.3 King's B medium

Subculture oxidase positive reddish brown cultures from 8.5.1 onto King's B medium and incubate up to 5 d at (36 ± 2) °C[3)]. Examine the growth under UV radiation daily and note the presence of any fluorescence. Record as positive any fluorescence appearing up to 5 d.

8.5.4 Acetamide broth

Inoculate a tube with the subculture from 8.5.1, and incubate at (36 ± 2) °C for (22 ± 2) h. Add 1 to 2 drops of Nessler reagent (5.2.5) and examine the tubes for the production of ammonia, characterized by the production of a colour varying from yellow to brick red depending upon concentration.

8.5.5 Enumeration

Count as confirmed *Pseudomonas aeruginosa* all colonies which produce pyocyanin (blue/green pigment) or which are oxidase positive, fluoresce under UV radiation (8.4 or 8.5.3) and are able to produce ammonia from acetamide (8.5.4).

NOTE Colonies which fluoresce on the primary membrane are invariably oxidase positive so they do not need to be tested for this parameter (see Table 1).

9 Expression of results

From the number of characteristic colonies counted on the membranes, and taking account of the proportion of confirmatory tests performed, calculate the number of confirmed *Pseudomonas aeruginosa* present in a specific volume of the water. For mineral water, spring water and other bottled waters, the volume will be 250 ml (see, e.g. References [1], [2] and [3] in the Bibliography). For other waters, the volume will usually be 100 ml.

3) Usually 24 h is sufficient.

EXAMPLE

If

— P is the number of blue/green colonies; all counted as confirmed target;

— F is the number of fluorescent colonies;

— R is the number of reddish brown colonies;

— n_F is the number of fluorescent colonies tested for ammonia production;

— c_F is the number of fluorescent colonies positive for ammonia production;

— n_R is the number of reddish brown colonies tested for ammonia and oxidase production and fluorescence on King's B;

— c_R is the number of reddish brown colonies positive for ammonia and oxidase production and fluorescence on King's B.

Then, the number of *Pseudomonas aeruginosa* is equal to $P + F(c_F/n_F) + R(c_R/n_R)$ per volume of sample examined.

Alternatively, express the results qualitatively by stating that *Pseudomonas aeruginosa* were present or absent in the volume of water examined.

10 Test report

The test report shall specify the following:

a) a reference to this International Standard (ISO 16266:2006);

b) all details necessary for complete identification of the sample;

c) the results obtained expressed in accordance with Clause 9;

d) any particular occurrence(s) observed during the course of the analysis and any operation(s) not specified in the method or considered optional, which may have influenced the results.

11 Performance data

In a trial in six laboratories from five countries, the following results were obtained (see Table 2).

Table 2 — *Pseudomonas* agar trial — Mean recoveries (%) relative to the count on nutrient agar after dilution in distilled water and filtration

Strain	Incubation	%
1	24 h	101,7
	48 h	100,1
2	24 h	92,6
	48 h	91,3
3	24 h	104,4
	48 h	124,8
4	24 h	94,7
	48 h	91,3

12 Interferences

Where large numbers of presumptive *Pseudomonas aeruginosa* are isolated, the spreading nature of colonies can hinder precise quantitative assessment.

13 Quality assurance

Pseudomonas aeruginosa NCTC 10332 can be used as positive control and *E. coli* NCTC 9001 as negative control for all stages.

Annex A
(informative)

Further information about *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the type species of the genus *Pseudomonas*.

It is a Gram negative, non-sporing rod which is oxidase and catalase positive. It exhibits oxidative metabolism as indicated by the Hugh and Leifson test, generally reduces nitrate beyond the stage of nitrite and produces ammonia from the breakdown of acetamide. Most strains (98 %) produce a water-soluble fluorescing pigment. The majority of strains are capable of growth at 42 °C but not at 4 °C which differentiates *Pseudomonas aeruginosa* from *Pseudomonas fluorescens* which grows at 4 °C but not 42 °C.

Gelatin is liquefied, casein is hydrolysed, but starch is not hydrolysed. The pigment pyocyanin (blue-green) is produced by more than 90 % of strains.

Annex B
(informative)

Alternative media

Alternative media to nutrient agar can be used, provided they are non-selective and do not contain a fermentable carbohydrate.

Bibliography

[1] Council Directive 80/777/EEC on the approximation of the laws of the Member States relating to the exploitation and marketing of natural mineral waters. *Official Journal of the European Communities,* **L229**, 1980, pp. 1-10

[2] Directive 96/70/EC of the European Parliament and of the Council amending Council Directive 80/777/EEC on the approximation of the laws of the Member States relating to the exploitation and marketing of natural mineral waters. *Official Journal of the European Communities*, **L299**, 1996, pp. 26-28

[3] Council Directive 98/83/EC on the quality of water intended for human consumption. *Official Journal of the European Communities*, **L330**, 1998, pp. 32-53

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