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# Water quality — Detection and enumeration of *Pseudomonas aeruginosa* —

## Part 2: Membrane filtration method

*Qualité de l'eau — Recherche et dénombrement de* Pseudomonas aeruginosa *—*

*Partie 2: Méthode par filtration sur membrane*



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## 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 8360-2 was prepared by Technical Committee ISO/TC 147, *Water quality*.

ISO 8360 consists of the following parts, under the general title *Water quality — Detection and enumeration of* Pseudomonas aeruginosa:

*Part 1: Method by enrichment in liquid medium*

*Part 2: Membrane filtration method*

Annex A of this part of ISO 8360 is for information only.





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## Introduction

*Pseudomonas aeruginosa* [1] may occur in water for a variety of reasons and from a variety of sources, but it cannot be used as an indicator of faecal pollution and the significance of its presence cannot always be precisely defined. However, since in certain circumstances it may be the cause of some opportunist infections in man, especially in debilitated patients, its presence in drinking water, bottled waters, swimming pools and hospital water supplies is considered undesirable.



1) See annex A for further information.

# Water quality — Detection and enumeration of *Pseudomonas aeruginosa* —

# Part 2: Membrane filtration method



## 1 Scope

This part of ISO 8360 presents a method for the isolation of *Pseudomonas aeruginosa* and the estimation of the numbers of this organism in water samples by the membrane filtration technique.

This method is applicable to all types of water provided that the amount of suspended particulate matter is not such that it prevents the passage of either water or nutrients through the membrane filter.

In water where the expected number of *Pseudomonas aeruginosa* is low, e.g. bottled water, or the water contains a relatively high level of residual disinfectant (e.g. swimming pools), the method described in ISO 8360-1 is recommended.

## 2 Normative references

The following standards contains provisions which, through reference in this text, constitute provisions of this part of ISO 8360. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 8360 are encouraged to investigate the possibility of applying the most recent editions of the standards listed below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696 : 1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-1 : 1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2 : 1982, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3 : 1985, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 6887 : 1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 8199 : 1988, *Water quality — General guide to the enumeration of micro-organisms by culture.*

## 3 Definition

For the purposes of this part of ISO 8360, the following definition applies.

*Pseudomonas aeruginosa:* Micro-organisms capable of growth on Drake's medium 19 selective media and producing characteristic colonies when grown on milk agar at 42 °C.

## 4 Principle

A measured volume of the water sample, or a dilution of the sample, is filtered through a membrane filter that has filtration characteristics equivalent to a rated pore diameter of 0,45 µm. The membrane filter is placed on the selective medium and incubated under the conditions specified for the medium.

### 4.1 Enumeration

The numbers of presumed *Pseudomonas aeruginosa* are obtained by counting the number of characteristic colonies on the membrane filter after incubation.

### 4.2 Confirmation

Subcultures are made from each membrane filter onto plates of milk agar medium. After incubation, the plates are examined for typical colonies of *Pseudomonas aeruginosa*.

#### 4.2.1 Non-pigmented and atypical strains

Subcultures are made either from milk agar plates or directly from the membrane filters onto solid agar media and incubated.

Pure cultures are obtained by further subculture onto plates of the same agar medium as required. Each pure culture is finally tested for certain biochemical characteristics (see annex A).

## 5 Diluents, culture media and reagents

Use reagents of analytical reagent quality in the preparation of culture media and diluents, unless otherwise specified. Prepare media using glass-distilled water, or water of equivalent quality, complying with ISO 3696 grade 3.

Alternatively, commercially available dehydrated media can be used. The media shall be prepared according to the manufacturer's instructions and selective agents added, as supplements at the given concentrations.

### 5.1 Dilution fluids

Use one of the diluents given in ISO 8199.

### 5.2 Culture medium

It is essential that the culture medium used be suitable for the type of water to be analysed and the purpose of the analysis. Use the following medium for the determination of presumed *Pseudomonas aeruginosa*.

### 5.3 Drake's medium 19

#### 5.3.1 Composition

| | |
|---|---|
| Peptone | 20 g |
| Ethanol | 25 ml |
| Anhydrous potassium sulfate | 10 g |
| Anhydrous magnesium chloride | 1,4 g |
| Hexadecyltrimethylammonium bromide (cetrimide) | 0,5 g |
| Water | to 1 000 ml |

#### 5.3.1.1 Preparation

Dissolve all the constituents in the water and proceed in either of the following ways.

Add the ethanol and distribute in sterile screw-capped bottles. Tighten the caps on the bottles to the point where the seal in the lid just begins to engage with the lip of the bottle. Autoclave at 121 °C ± 1 °C for 15 min. Tighten the caps on each bottle, immediately after removal from the autoclave, to prevent loss of ethanol by evaporation. Do not use polypropylene caps without seals.

Alternatively, sterilize the ethanol by filtration through a cellulose acetate or nitrate membrane of average pore size 0,22 µm and then add it aseptically to the medium after autoclaving and cooling. Adjust the pH to 7,2 ± 0,2. Store in screw-capped bottles in the dark at room temperature for up to a maximum of 3 months.

### 5.4 Confirmatory medium

#### 5.4.1 Milk agar with cetrimide

#### 5.4.1.1 Composition

| | |
|---|---|
| Skim milk powder | 100 g |
| Yeast extract broth (see below) | 250 ml |
| Agar | 15 g |
| Hexadecyltrimethylammonium bromide (cetrimide) | 0,3 g |
| Water | to 750 ml |

Yeast extract broth:

| | |
|---|---|
| Bacteriological yeast extract | 3 g |
| Bacteriological peptone | 10 g |
| Sodium chloride | 5 g |
| Water | to 1 000 ml |

#### 5.4.1.2 Preparation of medium

Prepare the yeast extract broth by dissolving all the constituents in the distilled water by steaming. Adjust the pH between 7,2 and 7,4. Sterilize by autoclaving at 121 °C ± 1 °C for 20 min.

Mix the sterile yeast extract broth, cetrimide and agar, and steam this mixture until the agar has dissolved. In a separate glass container, add the skim milk powder to the distilled water and mix, preferably with a magnetic stirrer, until the powder has completely dissolved. Autoclave both solutions separately at 121 °C ± 1 °C for 5 min. To prevent caramelization of the milk, take care to follow these instructions. Cool the solutions to 50 °C to 55 °C, aseptically add the milk solution to the agar medium and mix well.

#### 5.4.1.3 Preparation of agar plates

Distribute 15 ml portions of the final agar medium into sterile Petri dishes (see 6.1). Allow the medium to solidify in the plates. Dry the plates. Store at 4 °C ± 1 °C for a maximum of 1 month.

## 6 Apparatus and glassware

Usual microbiological laboratory equipment, and

### 6.1 Glassware

All glassware shall be sterilized at 170 °C ± 5 °C for 1 h in a dry oven or at 121 °C ± 1 °C for 15 min in an autoclave before use. Use sterile Petri dishes with a diameter of either 90 mm or 100 mm.

**6.2 Incubators,** capable of being maintained at 37 °C ± 1 °C and 42 °C ± 0,5 °C.

**6.3 Ultraviolet lamp** emitting light of wavelength 360 nm ± 20 nm.

## 7 Sampling

Carry out the collection of samples in accordance with ISO 5667-1, ISO 5667-2 and ISO 5667-3. The volume of sample collected shall be sufficient to carry out all the tests necessary, taking into account the expected numbers of *Pseudomonas aeruginosa* in the water under examination.

## 8 Procedure

Carry out the membrane filtration technique, and prepare the dilutions in accordance with ISO 8199 and ISO 6887.

### 8.1 Dilutions

Prepare 10-fold serial dilutions of the sample in a pre-sterilized diluent (5.1) in accordance with ISO 8199.

### 8.2 Membrane filtration

Filter volumes of the water sample or portions of the dilution through a sterile membrane filter with a rated pore diameter equivalent to 0,45 µm. In accordance with ISO 8199, place each membrane on a sterile filter pad saturated with Drake's medium 19, ensuring that no air is trapped beneath.

NOTE — Excess Drake's medium 19 should be removed from the Petri dish prior to placing the membrane on the filter pad.

### 8.3 Incubation of membranes

Incubate the Petri dishes at 37 °C ± 1 °C for 48 h in containers that prevent moisture loss. Examine the membranes for blue-green or greenish-brown colonies, or colonies which exhibit fluorescence under exposure to ultraviolet light in either a darkened room or apparatus which excludes visible light.

NOTE — Incubation at 42 °C ± 0,5 °C for up to 48 h may be used if the water samples are likely to contain large numbers of other aquatic bacteria.

The possible adverse effect of this procedure on the number of organisms recovered should be considered.

### 8.4 Confirmation

#### 8.4.1 Milk agar

Subculture the characteristic colonies from 8.3 onto the surface of milk agar plates. Incubate the milk agar plates at 42 °C ± 0,5 °C for 24 h. Examine the plates for growth, pigment production and casein hydrolysis (clearing of the milk medium around the colonies) and record the reactions as shown in table 1.

**Table 1 — *Pseudomonas aeruginosa* reactions**

| Reaction mode | Typical | Atypical *) | |
|---|---|---|---|
| | (1) | (2) | (3) |
| Casein hydrolysis | + | + | + |
| Growth at 42 °C ± 0,5 °C | + | + | + |
| Fluorescence (under UV irradiation only) | + | + | − |
| Pyocyanine (blue-green) pigment | + | − | − |

\+ = positive reaction

− = negative reaction

*) Other bacteria can sometimes give atypical reactions (2) and (3). In such instances the procedure described in 8.4.3 should be followed.

NOTE — Pigment production on the membranes may be inhibited by the growth of bacteria other than *Pseudomonas aeruginosa*. In such cases the milk agar plates should be exposed to daylight at room temperature before they are examined for pigment production.

#### 8.4.2 Enumeration

Count as confirmed *Pseudomonas aeruginosa* all colonies which exhibit the reactions (1) and (2) (see table 1 in 8.4.1).

Count as presumed *Pseudomonas aeruginosa* all colonies which show, after incubation, the following characteristics:

blue-green or greenish-brown coloration or exhibit fluorescence when exposed to ultraviolet light.

NOTE — Others identified as non-pigmented or atypical *Pseudomonas aeruginosa* by the procedure in 8.4.3 may be included also.

#### 8.4.3 Non-pigmented strains

NOTE — As a further step, it is possible to obtain confirmation of non-pigmented strains. If required, a suitable method is to take a loopful of culture medium and transfer it to a milk agar plate. The plate is incubated at a temperature of 37 °C ± 1 °C for 24 h. A well-isolated colony is selected and final confirmation is obtained by testing for certain biochemical characteristics (see annex A). Commercially available identification kits may be used.

## 9 Expression of results

From the number of characteristic colonies counted on the membranes, and taking account of the confirmatory tests performed, calculate the number of confirmed *Pseudomonas aeruginosa* present in 100 ml of water sample in accordance with ISO 8199.

Alternatively, express the results qualitatively by stating that *Pseudomonas aeruginosa* were present or absent in 100 ml of water sample.

When larger volumes are examined, e.g. bottled waters, express the results as numbers per volume examined.

## 10 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 8360;

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

c) where applicable, the confirmation methods used to identify non-pigmented strains;

d) the results obtained expressed in accordance with clause 9;

e) any particular occurence(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.

# 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 is capable of growth at 42 °C but not at 4 °C; it usually produces a water soluble fluorescing pigment (98 % of strains) and exhibits oxidative metabolism as indicated by the Hugh and Leifson test. It generally reduces nitrate beyond the stage of nitrite and produces ammonia from the breakdown of acetamide.

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