SLOVENSKI STANDARD
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Kakovost vode - Ugotavljanje prisotnosti in števila Pseudomonas aeruginosa - 2. del: Metoda najverjetnejšega števila (ISO 16266-2:2018)

Water quality - Detection and enumeration of Pseudomonas aeruginosa - Part 2: Most probable number method (ISO 16266-2:2018)

Wasserbeschaffenheit - Nachweis und Zählung von Pseudomonas aeruginosa - Teil 2: Verfahren zur Bestimmung der wahrscheinlichsten Keimzahl (ISO 16266-2:2018)

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Qualité de l'eau - Recherche et dénombrement de Pseudomonas aeruginosa - Partie 2: Méthode du nombre le plus probable (ISO 16266-2:2018)

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INTERNATIONAL STANDARD

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Water quality — Detection and enumeration of Pseudomonas aeruginosa —

Part 2: Most probable number method

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

Partie 2: Méthode du nombre le plus probable

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbibiological methods*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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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, for example, Council Directive 2009/54/EC, Reference [1]). 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, Reference [2]). Other waters, including swimming and spa pool waters, water for human consumption and hospital waters, may sometimes be tested for *Pseudomonas aeruginosa* for reasons of public health. In these cases, it is typical to examine 100 ml volumes.

The method described in this document can be applied to a range of types of water, for example, hospital waters, drinking water and non-carbonated bottled waters intended for human consumption, groundwater, swimming pool and spa pool waters including those containing high background counts of heterotrophic bacteria (see References [3], [4], [5], [6] and [7]).

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Water quality — Detection and enumeration of *Pseudomonas aeruginosa* — Part 2: Most probable number method

WARNING — Persons using this document should be familiar with normal laboratory practice. This document 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.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document are carried out by suitably qualified staff.

1 Scope

This document specifies a method for the enumeration of *Pseudomonas aeruginosa* in water. The method is based on the growth of target organisms in a liquid medium and calculation of the most probable number (MPN) of organisms by reference to MPN tables.

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This document is applicable to a range of types of water. For example, hospital waters, drinking water and non-carbonated bottled waters intended for human consumption, groundwater, swimming pool and spa pool waters including those containing high background counts of heterotrophic bacteria.

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This document does not apply to carbonated bottled waters, flavoured bottle waters, cooling tower waters or marine waters, for which the method has not been validated. These waters are, therefore, outside the scope of this document. Laboratories can employ the method presented in this document for these matrices by undertaking appropriate validation of performance of this method prior to use.

The test is based on a bacterial enzyme detection technology that signals the presence of *P. aeruginosa* through the hydrolysis of a 7-amino-4-methylcoumarin aminopeptidase substrate present in a special reagent. *P. aeruginosa* cells rapidly grow and reproduce using the rich supply of amino acids, vitamins and other nutrients present in the reagent. Actively growing strains of *P. aeruginosa* have an enzyme that cleaves the 7-amido-coumarin aminopeptidase substrate releasing a product which fluoresces under ultraviolet (UV) light. The test described in this document provides a confirmed result within 24 h with no requirement for further confirmation of positive wells.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 8199, *Water quality — General guide to the enumeration of micro-organisms by culture*

ISO 11133, *Microbiology of food, animal feeding stuffs, food production, environment and water — Preparation, production, storage and performance testing of culture media*

ISO 19458, *Water quality — Sampling for microbiological analysis*

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ISO/IEC Guide 2, *Standardization and related activities — General vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/IEC Guide 2 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
Pseudomonas aeruginosa
species of microorganism that is capable of growing in a selective broth and capable of hydrolyzing a diagnostic 7-amino-4-methylcoumarin aminopeptidase substrate present in the reagent

Note 1 to entry: See Annex A for further information on *P. aeruginosa*.

4 Principle

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A snap pack of dehydrated medium is added to a sample of water (100 ml or 250 ml), or to a dilution of a sample made up to 100 ml. Sample plus medium is gently shaken to ensure adequate mixing and to afford dissolution of the medium. When enumeration is required, the sample plus medium (100 ml) is then aseptically poured into either a Quanti-Tray[1)] or Quanti-Tray/2000[1)] to enumerate up to 201 organisms or 2 419 organisms respectively per 100 ml sample. The procedure for the enumeration of 250 ml samples is described in 8.2. Trays are sealed with a Quanti-Tray[1)] Sealer. Quanti-Trays[1)] or vessels (for presence/absence tests) are then incubated at (38 ± 0,5) °C for 24 h to 28 h. Results are confirmed at 24 h but may be read up to 28 h.

After incubation, vessels or Quanti-Tray[1)] sample wells that exhibit any degree of blue fluorescence under long wavelength ultraviolet light (365 nm) are considered positive for *P. aeruginosa*.

By means of statistical tables, or a simple computer program, the MPN of *P. aeruginosa* in 100 ml or 250 ml of the sample can be determined.

This method is also suitable as a qualitative procedure.

5 Apparatus and glassware

Usual microbiological laboratory equipment, and, in particular, the following equipment.

[1)] Quanti-Tray is a trademark or registered trademark of IDEXX Laboratories, Inc. or its affiliates in the United States and/or other countries. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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5.1 Apparatus for sterilization by steam (autoclave).

Apparatus and glassware not supplied sterile shall be sterilized according to the instructions given in ISO 8199.

5.2 Hot air oven, for dry heat sterilization.

5.3 Incubator, thermostatically controlled at (38 ± 0,5) °C.

5.4 Quanti-Tray[1] sealer and rubber insert.

5.5 Sterile wide mouthed vessels of at least 110 ml.

5.6 Ultraviolet lamp, 365 nm, 6 watt, long wavelength.

5.7 Quanti-Tray[1] or Quanti-Tray/2000[1], in accordance with Annex B.

6 Culture media, diluents and reagents

6.1 Basic materials

The method utilises Pseudalert[2], a medium available as a ready-to-use powder dispensed in snap packs. Each snap pack contains sufficient medium (2,45 g for 100 ml samples) for a single test. For quantitative enumeration of 250 ml samples, one snap pack of 2,45 g is added to each aliquot of divided sample as described in 8.2. The Pseudalert[2] reagent should be tan in colour and free flowing. The medium contains nutrients such as amino acids and vitamins, buffer, sodium chloride, magnesium sulfate, growth indicators, antibiotics and nitrogen sources. The medium is stored under ambient conditions (2 °C to 30 °C) out of direct sunlight and should be used before the expiry date listed on the snap pack. The reagent has a shelf-life of 12 months from the date of manufacture.

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The composition of the Pseudalert[2] medium shall be in accordance with Annex C. Performance characteristics for this method are provided in Annex D.

6.2 Diluent

For dilutions to be used with Pseudalert[2], use only sterile, non-inhibitory, oxidant-free water. The use of buffered, saline or peptone-containing diluents interferes with the performance of the test.

6.3 Antifoam B

Antifoam B used as a 1 % active, water soluble suspension of silicone. This reagent is added to samples in order to minimize the formation of air bubbles during mixing.

NOTE Vessels already containing antifoam are available.

[2] Pseudalert is a trademark or registered trademark of IDEXX Laboratories, Inc. or its affiliates in the United States and/or other countries. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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7 Sampling

Carry out the collection, preservation and handling of samples as specified in ISO 19458.

8 Procedure

8.1 Transport and storage of the samples

Samples should be transported and stored in accordance with ISO 19458. Analysis should be commenced on the day of collection or within 12 h. Under exceptional circumstances, the samples may be kept at (5 ± 3) °C for up to 24 h prior to examination. In this case, the storage time shall be reported in the test report.

8.2 Preparation of the sample and inoculation of media

8.2.1 Preparation of 100 ml samples

For enumeration of 100 ml samples, aseptically add a single snap pack of Pseudalert2) medium to each 100 ml volume of sample or dilution of sample in a sterile, transparent vessel and mix well. When the medium is completely dissolved, the sample plus medium is aseptically poured into either a Quanti-Tray1) or Quanti-Tray/20001) and then sealed with the Quanti-Tray1) Sealer. In order to minimize air bubbles within wells, samples can be prepared in pre-sterilized vessels containing antifoam B. Alternatively, antifoam B can be added to each vessel using a dropper bottle. An alternative MPN method is where the water sample in which the Pseudalert2) has been dissolved is distributed into sterile tubes for determination of the MPN using a more traditional MPN format (e.g. 1 × 50 ml and 5 × 10 ml).

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NOTE The presence of high mineral content (especially magnesium and/or calcium) can cause the Pseudalert2) reagent mixture to become cloudy but this does not affect the outcome of the test.

8.2.2 Preparation of 250 ml samples

For enumeration of 250 ml samples, divide the sample into three sterile, transparent vessels with two samples having aliquots of 100 ml and one sample having an aliquot of 50 ml. Make the 50 ml sample up to 100 ml by the addition of 50 ml of sterile, non-inhibitory, oxidant-free water. Aseptically add a single snap pack of Pseudalert2) medium to each of the three 100 ml volumes of sample and mix well. When the medium is completely dissolved, the three 100 ml volumes of sample plus medium are each aseptically poured into three separate Quanti-Tray1) or Quanti-Tray/20001) and then sealed with the Quanti-Tray1) Sealer. In order to minimize air bubbles within wells, samples can be prepared in pre-sterilized vessels containing antifoam B. Alternatively, antifoam B can be added to each vessel using a dropper bottle. Appropriate labelling of the three Quanti-Tray1) where the tray containing the 50 ml portion of the sample (i.e. the diluted portion) is clearly distinguishable from the two trays containing the 100 ml undiluted portions of the sample is essential. This is important for the correct calculation of the final count.

8.3 Incubation and differentiation

Incubate the inoculated Quanti-Tray1) for 24 h to 28 h at (38 ± 0,5) °C for *P. aeruginosa*.

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8.4 Examination of results

Examine the Quanti-Tray[1)] or Quanti-Tray/2000[1)] after incubation under UV irradiation (365 nm) in a dark room or in a chamber that obscures ambient light. Ensure that the UV light is facing away from your eyes and toward the sample. The efficacy of the UV lamp should be checked regularly using a fluorescence positive control in accordance with Clause 10. The lamp should also be replaced according to the manufacturer's stated lamp life (e.g. 6 000 h) or annually, whichever is sooner. Regard and count any wells that exhibit any degree of blue fluorescence as positive for *P. aeruginosa*. For interpretation purposes compare with a negative control. If there is doubt about the fluorescence for a well at 24 h, return the tray to the incubator for further incubation without exceeding a maximum incubation time of 28 h. Placing the Quanti-Tray[1)] or Quanti-Tray/2000[1)] rubber insert over the sample can facilitate identification of fluorescing wells.

9 Expression of results

From the number of wells on a Quanti-Tray[1)] or Quanti-Tray/2000[1)] that are positive, the MPN/100 ml for *P. aeruginosa* can be calculated by reference to statistical tables or by using a computer MPN generator program, see Tables B.1 and B.2. For enumeration from 250 ml samples the MPN is calculated using the sum of the counts from the two Quanti-Tray[1)] containing undiluted portions of the sample as one count and the count from the Quanti-Tray[1)] containing the diluted portion of sample as the second count, see Table B.3.

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10 Quality assurance

The laboratory shall have a clearly defined quality control system to ensure that the apparatus, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks is part of the test.

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For the definition of productivity and selectivity refer to ISO 11133. The performance of Pseudalert[2)] shall be tested according to the methods and criteria described in ISO 11133 (see Table 1).

Table 1 — Performance testing of Pseudalert[2)]

Function	Incubation	Control strain[a]	Reference medium	Method of control	Criteria	Characteristic reactions
Productivity	24 h to 28 h/ (38 ± 0,5) °C	*P. aeruginosa* WDCM 00024 or WDCM 00025	TSA	Quantitative	PR ≥ 0,5	Blue fluorescence
Selectivity	24 h to 28 h/ (38 ± 0,5) °C	*P. fluorescens* WDCM 00115	—	Qualitative	Total inhibition	No blue fluorescence

[a] Refer to the reference strain catalogue available on http://www.wfcc.info/pdf/WDCM_Reference_Strain_Catalogue.pdf on culture collection strain numbers and contact details.

11 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 16266-2:2018;

b) all information required for the complete identification of the sample;

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c) the results 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 this document that may have influenced the results.

If, under exceptional circumstances, the sample was kept at (5 ± 3) °C for up to 24 h prior to examination, the storage time shall be reported in the test report.

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Annex A
(informative)

Further microbiological information about *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the type species of the genus *Pseudomonas* which is the type genus of the family Pseudomonadaceae of the order Pseudomonadales.

It is a Gram negative, non-spore forming 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 able to grow at 42 °C but not at 4 °C which differentiates *P. aeruginosa* from *P. fluorescens* which grows at 4 °C but not at 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 of *P. aeruginosa*.

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