International Standard 6461/2

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Water quality — Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) — Part 2: Method by membrane filtration

Qualité de l'eau — Recherche et dénombrement des spores de micro-organismes anaérobies sulfito-réducteurs (clostridia) — Partie 2 : Méthode par filtration sur membrane

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Foreword

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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 6461/2 was prepared by Technical Committee ISO/TC 147, *Water quality*.

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Water quality — Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) — Part 2: Method by membrane filtration

0 Introduction

The spores of sulfite-reducing anaerobes (clostridia) are widespread in the environment. They are present in human and animal faecal matter, in waste water and in soil. Unlike *Escherichia coli* and other coliform organisms, the spores survive in water for long periods as they are more resistant than vegetative forms to the action of chemical and physical factors. They may thus give an indication of remote or intermittent pollution. They may even be resistant to chlorination at levels which are normally used for the treatment of water, and they are thus useful for control purposes.

ISO 6461 consists of the following parts:

Part 1: Method by enrichment in a liquid medium.

Part 2: Method by membrane filtration.

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1 Scope

This part of ISO 6461 specifies a method for the detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) by membrane filtration.

2 Field of application

The method can be applied to all types of water, except when a large amount of particulate material is liable to be retained by the membrane.

3 References

ISO 3696, *Water for laboratory use — Specifications.*

ISO 5667, *Water quality — Sampling —*

Part 2: Guidance on sampling techniques.

Part 3: Guidance on the preservation and handling of samples.

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

ISO 8199, *Water quality — General guidance for microbiological examination by enumeration of micro-organisms on culture media.*[1)]

4 Definition

For the purpose of this part of ISO 6461, the following definition applies.

clostridia: Sulfite-reducing, spore-forming, anaerobic micro-organisms which belong to the Bacillaceae family and the genus *Clostridium.*

5 Principle

The detection of spores of sulfite-reducing anaerobes (clostridia) in a specified volume of a water sample requires the following steps.

5.1 Selection of spores

Selection of spores in the sample by applying heat for a period of time sufficient to destroy vegetative bacteria.

5.2 Membrane filtration and culture

Filtration of the water sample through a membrane filter having a pore size such that bacterial spores (0,2 µm) are retained in or on the membrane filter.

Placing of the filter on a specialized selective culture medium (sulfite-iron-agar), followed by incubation at 37 ± 1 °C for 20 ± 4 h and 44 ± 4 h, and counting of any black colonies.

6 Culture media and reagents

6.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluents and culture media, dehydrated basic components or complete dehydrated media be used. Similarly, commercially prepared reagents may also be used. The manufacturer's instructions shall be rigorously followed.

1) At present at the stage of draft.

The chemical products used for the preparation of the culture media and the reagents shall be of recognized analytical quality.

The water used shall be distilled or deionized water, free from substances that might inhibit the growth of micro-organisms under the test conditions (see ISO 3696).

Measurements of pH shall be made using a pH meter, measurements being referred to a temperature of 25 °C.

If the prepared culture media are not used immediately, they shall, unless otherwise stated, be stored in the dark at approximately 4 °C, for no longer than 1 month.

6.2 Sulfite-iron-agar

6.2.1 Basal medium (nutrient agar)

Composition

Meat extract	3 g
Peptone	10 g
Sodium chloride (NaCl)	5 g
Agar	15 g
Water	1 000 ml

Preparation

Steam to dissolve, make up to 1 litre with water, and adjust the pH to 7,6 ± 0,1 with 1 mol/l sodium hydroxide solution. Sterilize at 121 ± 1 °C in the autoclave for 20 min.

Store in the refrigerator after solidifying.

6.2.2 Sodium sulfite (Na_2SO_3) solution.

Dissolve 10 g of sodium sulfite in 100 ml of water.

It is advisable to prepare a fresh solution every two weeks.

6.2.3 Iron(II) sulfate ($FeSO_4$) solution.

Dissolve 8 g of crystallized iron(II) sulfate in 100 ml of water.

6.2.4 Complete medium

Immediately before use, melt the basal medium (6.2.1) and to each 18 ml volume add 1 ml of sodium sulfite solution (6.2.2) and five drops of iron(II) sulfate solution (6.2.3).

Add 1 ml of the sodium sulfite solution and 5 drops of the iron(II) sulfate solution to the agar tubes just before the embedding procedure (see 9.3).

6.3. Tryptose-sulfite-agar (alternative medium)

Composition

Tryptose	15 g
Soytone	5 g
Yeast extract	5 g
Sodium metabisulfite	1 g
Ammonium iron(III) citrate	1 g
Water	1 000 ml

Preparation

Steam to dissolve, and adjust the pH to 7,6 ± 0,1 at 25 °C.

Distribute into test tubes in volumes of 18 ml. Sterilize the medium for 15 min at 121 ± 1 °C.

Store in the refigerator at 4 to 5 °C.

Discard unused medium 2 weeks after preparation.

7 Apparatus and glassware

Usual microbiological laboratory equipment, and

7.1 Glass flasks (Erlenmeyer flask, round-bottom flask, or conical flask), of capacity 2 litres.

7.2 Test tubes, 160 mm × 16 mm.

7.3 Graduated pipettes, of capacity 10 ml, graduated in divisions of 0,1 ml.

7.4 Volumetric pipettes, of capacity 10 ml.

7.5 Jars, of capacity 1 litre.

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7.6 Steamer.

7.7 Water bath.

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7.8 Membrane filtration apparatus.

7.9 Sterile membrane filters, of nominal pore size 0,2 µm.

NOTE — The quality of membrane filters may vary according to brand or even from batch to batch. It is therefore advisable to check the quality on a regular basis according to ISO 7704.

7.10 Incubator, capable of being maintained at 37 ± 1 °C.

7.11 Petri dishes.

8 Sampling

Refer to ISO 5667/2 and ISO 8199 for sampling techniques.

9 Procedure

9.1 Treatment of samples

Refer to ISO 5667/3 for guidance on the preservation and handling of samples, and to ISO 8199.

9.2 Selection of spores (technique)

Before the test, the sample of water should be heated in a water bath at 75 ± 5 °C for 15 min from the time it reaches that temperature. A similar bottle containing the same volume

of water as the test sample should be used periodically as a control in order to check the heating time required. The temperature of the water in the control bottle can be constantly recorded by thermometer.

9.3 Inoculation and incubation

Refer to ISO 7704 for a general description of the membrane filtration technique.

According to the instructions in that document, filter a suitable volume of water. For drinking water, spring and well water, mineral waters, sea-water, and surface water, less polluted with clostridia, filter 100 ml; for highly polluted water or sewage use smaller volumes. Volumes of water smaller than 10 ml should be mixed with 10 to 100 ml of sterile water or diluent.

Adjust the dilutions so that any resultant black colonies are well separated and can be easily counted.

After filtration, remove the membrane with sterile forceps and place it face downwards on the bottom of a Petri dish, ensuring that no air bubbles are trapped under the filter. Then carefully pour 18 ml of the liquefied complete culture medium, previously cooled to about 50 °C, over the membrane while holding it still with sterile forceps. After this layer of medium has set, incubate anaerobically or under other conditions which ensure anaerobiosis at a temperature of 37 ± 1 °C for 20 ± 4 h and 44 ± 4 h. If an anaerobic jar or an anaerobic incubator is used, the membrane filter may be placed on the surface of the agar face upwards.

9.4 Interpretation

Count all black colonies after incubation for 20 ± 4 h and 44 ± 4 h.

10 Expression of results

Express the results in accordance with ISO 8199.

11 Test report

The test report shall state the method used and express the results as the number of sulfite-reducing anaerobes (clostridia) per volume of sample. The 44 ± 4 h count should normally be reported. If this is not possible, the count at 20 ± 4 h should be reported as approximate only.

The test report shall also mention any operating details not specified in this part of ISO 6461, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

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