International Standard 6461/1

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Water quality — Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) — Part 1: Method by enrichment in a liquid medium

Qualité de l'eau — Recherche et dénombrement des spores de micro-organismes anaérobies sulfito-réducteurs (clostridia) — Partie 1: Méthode par enrichissement dans un milieu liquide

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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.

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Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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Water quality — Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) — Part 1: Method by enrichment in a liquid medium

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.

1 Scope

This part of ISO 6461 specifies a method for the detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) by enrichment in a liquid medium.

2 Field of application

The method is applicable to all types of water, including turbid water.

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 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 Enrichment culture

Detection and enumeration of spores of sulfite-reducing anaerobes by inoculating volumes of the sample into liquid enrichment media, followed by incubation at 37 ± 1 °C for 44 ± 4 h in anaerobic conditions.

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.

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

1) At present at the stage of draft.

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 Culture media and diluent

6.2.1 Diluent

Use one of the diluents given in ISO 8199.

6.2.2 Differential reinforced clostridial medium (DRCM)

6.2.2.1 Single strength basal medium

Composition

Peptone tryptic digest of meat	10 g
Meat extract	10 g
Yeast extract	1,5 g
Starch	1 g
Hydrated sodium acetate	5 g
Glucose	1 g
L-Cysteine-hydrochloride	0,5 g
Water	1 000 ml

Preparation

Mix the peptone, meat extract, sodium acetate and yeast extract with 800 ml of water.

With the remaining 200 ml of distilled water, prepare a starch solution as follows: mix the starch in a little cold water to form a paste. Heat the rest of the water to boiling point and slowly add it to the paste with constant stirring.

Then add this starch solution to the first mixture and heat to boiling point until it dissolves.

Finally, add the glucose and L-cysteine hydrochloride. Dissolve.

Adjust the pH to 7,1 to 7,2 with 1 mol/l sodium hydroxide.

Transfer 25 ml aliquots of the medium into screw-capped bottles of capacity 25 ml. Sterilize in the autoclave at 121 ± 1 °C for 15 min.

6.2.2.2 Double strength basal medium

Prepare the double strength medium as in 6.2.2.1 but reduce the volume of water by half.

Transfer 10 ml and 50 ml aliquots of the medium into screw-capped bottles of capacities 25 ml and 100 ml respectively.

6.2.3 Sodium sulfite (Na_2SO_3), 4 % (m/m) solution.

Dissolve 4 g of anhydrous sodium sulfite in 100 ml of water. Sterilize by filtration.

Store at between 2 and 5 °C.

It is advisable to prepare a fresh solution every 14 days.

6.2.4 Iron(III) citrate ($C_6H_5O_7Fe$), 7 % (m/m) solution.

Dissolve 7 g of iron(III) citrate in 100 ml of water. Sterilize by filtration.

Store at between 2 and 5 °C.

It is advisable to prepare a fresh solution every 14 days.

6.2.5 Complete medium

6.2.5.1 On the day of analysis, mix equal volumes of the solutions of sodium sulfite (6.2.3) and iron(III) citrate (6.2.4).

6.2.5.2 Add 0,5 ml of the mixture (6.2.5.1) to each bottle of single strength medium (6.2.2.1), which has been freshly heated and cooled.

6.2.5.3 Add 0,4 ml of the mixture (6.2.5.1) to each 10 ml, and 2 ml to each 50 ml, of double strength medium (6.2.2.2) similarly treated.

7 Apparatus and glassware

Usual microbiological laboratory equipment, and

7.1 Screw-cap bottles or **vials** and **stoppers** of boron silicate glass of capacities 200, 100 and 25 ml.

7.2 Volumetric pipettes, of capacities 10 and 1 ml.

7.3 Water baths, thermostatically controlled.

7.4 Test tubes, 150 mm × 13 mm.

7.5 Iron wire.

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

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

Add 50 ml of sample (9.2) to a 100 ml screw-cap bottle containing 50 ml of the double strength complete medium (6.2.5.3).

Add 10 ml of sample (9.2) to a series of five 25 ml screw-cap bottles containing 10 ml of double strength complete medium (6.2.5.3).

Add 1 ml of sample (9.2) to a series of five 25 ml screw-cap bottles containing 25 ml of single strength complete medium (6.2.5.2).

If necessary, add 1 ml of a 1 → 10 dilution of the sample (9.2) to a series of five 25 ml screw-cap bottles containing 25 ml of single strength complete medium (6.2.5.2).

In order to carry out a qualitative examination of 100 ml of drinking water or bottled water without making an MPN count, use a 200 ml vial filled with a mixture of 100 ml of double strength complete medium (6.2.5.3) and 100 ml of sample (9.2).

If necessary, top up all the bottles with the single strength complete medium (6.2.5.2) to bring the volume of liquid level with the neck and to ensure that only a very small volume of air remains, then seal the bottles hermetically, or incubate under anaerobic conditions.

Incubate the inoculated bottles at 37 ± 1 °C for 44 ± 4 h.

Large volumes of culture in hermetically sealed glass bottles may explode due to gas production. The addition of iron wire, heated to redness and placed into the medium before inoculation, may aid anaerobiosis.

9.4 Interpretation

Bottles in which blackening is observed, as a result of the reduction of sulfite and the precipitation of iron(II) sulfide, shall be regarded as positive.

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 most probable number of sulfite-reducing anaerobes (clostridia) per volume of sample. It 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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