

Australian Standard®

AS 4276.17.2:2016

Water microbiology

Method 17.2: Spores of *Clostridium perfringens*—Estimation of most probable number (MPN) using the multiple tube dilution technique

PREFACE

This Standard was prepared by the Standards Australia Committee FT-020, Water Microbiology, to supersede AS/NZS 4276.17.2:2000, *Water microbiology, Method 17.2: Spores of sulfite-reducing anaerobes (clostridia) including Clostridium perfringens—Estimation of most probable number (MPN) using the multiple tube dilution technique*.

The objectives of this revision are—

- (a) to incorporate an additional more selective medium [oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar] for testing of samples with a greater proportion of competing microflora and to incorporate other technical variations;
- (b) to incorporate culture media, reagents and to remove reference to AS 4276.2;
- (c) to update reference cultures; and
- (d) to update the references.

There is no ISO Standard for estimation of most probable number (MPN) using the multiple tube dilution technique for spores of *Clostridium perfringens*.

The term ‘informative’ has been used in this Standard to define the application of the appendix to which it applies. An ‘informative’ appendix is only for information and guidance.

FOREWORD

The spores of sulfite-reducing anaerobes such as *Clostridium perfringens* are widespread in the environment. They are present in human and animal faecal material, in waste water and in soil. Unlike *Escherichia coli* and other coliform bacteria, spores of *Clostridium perfringens* survive in water for months as they are more resistant to the action of chemical agents (e.g. chlorination) and physical conditions (e.g. heat) than vegetative forms and may be used as indicators of water quality.

As *Clostridium perfringens* is associated with faecal contamination, it can indicate remote or intermittent pollution in the absence of other faecal indicator microorganisms.

Monitoring of *C. perfringens* has proven useful for the assessment of the quality of water resources and to check the stages of water treatment to evaluate treatment efficiency.

METHOD

1 SCOPE

This Standard sets out a method for determining the most probable number (MPN) of spores of *C. perfringens* in water, using a multiple tube dilution technique, with a confirmation procedure for identifying *C. perfringens*.

Refer to AS/NZS 4276.1 for a description of the general procedure for estimating the MPN of microorganisms.

MPN procedures may be used for the examination of all water samples and are particularly applicable for samples containing sufficient suspended material to make filtration procedures impractical.

NOTE: A flow diagram of the procedure is shown in Appendix A.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS

2031 Water quality—Sampling for microbiological analysis

5013 Food microbiology

5013.16 Method 16: Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of *Clostridium perfringens*—Colony-count technique

AS/NZS

4276 Water microbiology

4276.1 Method 1: General information and procedures(ISO 8199:2005, MOD)

3 DEFINITIONS

For the purposes of this Standard the following definitions apply:

3.1 Presumptive *C. perfringens*

Bacteria that produce all shades of black or grey to yellow-brown colonies, even if the colour is faint, after anaerobic incubation at $36 \pm 2^\circ\text{C}$ or $44 \pm 1^\circ\text{C}$ for 21 ± 3 h, on tryptose-sulfite-cycloserine (TSC) agar at $44 \pm 1^\circ\text{C}$ or oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar at $36 \pm 2^\circ\text{C}$ or $44 \pm 1^\circ\text{C}$.

3.2 Confirmed *C. perfringens*

Anaerobic bacteria that produce characteristic colonies on TSC or OPSP agar, capable of growth at $44 \pm 1^\circ\text{C}$, and possess the enzyme acid phosphatase.

4 PRINCIPLE

A series of dilutions are inoculated into the differential reinforced clostridial medium (DRCM) in accordance with the MPN method in AS/NZS 4276.1. Tubes are incubated anaerobically at $44 \pm 1^\circ\text{C}$ for 48 ± 4 h. A loopful of broth from each blackened DRCM tubes is subcultured onto TSC or OPSP medium to obtain isolated colonies for confirmation.

This method consists of the following stages:

- (a) A heating procedure to eliminate non-spore-forming microorganisms from the test sample.
- (b) A multiple tube dilution cultural procedure to selectively detect H_2S producing anaerobic microorganisms in the heat treated sample.

- (c) A confirmation procedure to identify *C. perfringens* amongst the H₂S producing microorganisms.

Incubation at 44°C increases the selectivity of the test for *C. perfringens*.

The MPN is determined by reference to an MPN table and the result is reported as the most probable number per 100 mL.

5 MEDIA, REAGENTS, AND REFERENCE CULTURES

5.1 Culture media (see Appendix B)

5.1.1 Differential reinforced clostridial medium (DRCM)

DRCM should be distributed in screw-capped bottles instead of tubes, with sufficient depth of medium to ensure anaerobiosis during incubation.

5.1.2 Tryptose sulfite cycloserine (TSC) agar

5.1.3 Oleandomycin polymyxin sulphadiazine perfringens (OPSP) agar

5.1.4 Columbia blood agar or other suitable nutrient-rich agar

5.1.5 Columbia blood agar with neomycin (0.01%)

5.1.6 Acid phosphatase reagent

5.2 Reference cultures

5.2.1 Positive culture

C. perfringens ATCC 13124, NCTC 8237, WDCM 00007 or a culture traceable to any of these strains.

5.2.2 Negative culture

C. sporogenes ATCC 19404, WDCM 00008 or a culture traceable to any of these strains.

5.2.3 Use of reference cultures

The reference cultures are used in media quality control and analytical quality control procedures relevant to this method. The purpose of the reference cultures is to demonstrate and ensure that typical growth characteristics and test reactions are exhibited by the cultures on the media and in the tests used in this method.

When testing a sample of water by this standard method, a culture of the positive reference microorganism (5.2.1) shall be submitted to the test procedures at the same time to demonstrate and ensure that typical growth characteristics and test reactions are exhibited. The negative reference microorganism (5.2.2) is used in addition to the positive reference microorganism for the acid phosphatase test.

If the reference cultures do not give appropriate results, then an investigation shall be undertaken. Test results shall be regarded as invalid unless the investigation reveals reasons that invalidate the quality control procedures, e.g. prepared suspension shown not to contain target bacteria.

NOTE:

ATCC—American Type Culture Collection

NCTC—National Collection of Type Cultures

WDCM—World Data Centre for Microorganisms

6 APPARATUS

6.1 Screw cap containers

Suitable for the volume of water to be tested.

6.2 Pipettes or equivalent measuring devices

1 mL, 10 mL, 50 mL

6.3 Anaerobic incubators/jar

With appropriate equipment and materials to generate an atmosphere of approximately 90% hydrogen and 10% carbon dioxide.

6.4 Incubators or water bath

Able to maintain at $36 \pm 2^\circ\text{C}$, $44 \pm 1^\circ\text{C}$ and $60 \pm 2^\circ\text{C}$.

7 SAMPLES

Sample holding and storage times shall be in accordance with AS 2031.

8 TEST PROCEDURES

The procedure shall be as follows:

- (a) Heat the sample to $60 \pm 2^\circ\text{C}$ in a waterbath and maintain this temperature for 15 ± 1 min. The volume heated should be greater than the volume to be analysed. The temperature should be monitored by placing an appropriate thermometer in a reference bottle of the same size as the sample bottle and containing the same volume of water at the same initial temperature as the sample being treated. The time taken to reach $60 \pm 2^\circ\text{C}$ shall not exceed 15 min and can be minimized by ensuring the water in the water bath is circulated to maximize heat exchange.
- (b) Using DRCM heated by bringing to the boil or steaming for 5 minutes to drive off oxygen and cooled immediately before use, prepare and inoculate bottles with appropriate volumes dilutions of the heated sample, as described for the MPN method in AS/NZS 4276.1. Top-up with single strength medium so as to leave a minimal amount of air space.
- (c) Incubate the bottles at $44 \pm 1^\circ\text{C}$ for 48 ± 4 h. A positive reaction is shown by blackening of the medium.

NOTE: Large volumes of culture in sealed bottles may explode. Bottles may be sealed in plastic bags in order to contain any breakage or spillage during incubation. Alternatively, the bottles may be incubated anaerobically with the caps loosened and with sufficient headspace to avoid explosions.

9 CONFIRMATION OF *C. PERFRINGENS*

9.1 Waters with low levels of competing spore forming bacteria

The procedure shall be as follows: Streak a loopful of broth from blackened DRCM tubes onto TSC agar plates to obtain single colonies and incubate anaerobically at $44 \pm 1^\circ\text{C}$ for 21 ± 3 h.

9.2 Waters with high levels of competing spore forming bacteria

The procedure shall be as follows: Streak a loopful of broth from blackened DRCM tubes onto OPSP agar plates to obtain single colonies and incubate anaerobically at $36 \pm 2^\circ\text{C}$ for 21 ± 3 h.

When interpretation of results becomes problematic due to interference from spreading micro-organisms or unacceptable confirmation rates (presumptive *C. perfringens* colonies not confirming), or when samples have a history of problematic results, OPSP agar may be incubated at $44 \pm 1^\circ\text{C}$.

NOTE: Increasing the selectivity of a procedure may not recover some strains of the target microorganism.