BS ISO 14189:2013

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Water quality — Enumeration of *Clostridium perfringens* — Method using membrane filtration

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee EH/3/4, Microbiological methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Published by BSI Standards Limited 2013

ISBN 978 0 580 70332 4

ICS 07.100.20

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 November 2013.

Amendments issued since publication

Date	Text affected

BS ISO 14189:2013

INTERNATIONAL STANDARD

ISO 14189

First edition
2013-11-01

Water quality — Enumeration of *Clostridium perfringens* — Method using membrane filtration

Qualité de l'eau — Dénombrement de Clostridium perfringens — *Méthode de filtration sur membrane*

Reference number
ISO 14189:2013(E)

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Published in Switzerland

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Foreword

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The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Introduction

Clostridium perfringens is widely recognized as a valuable indicator for faecal pollution. Within the intestinal tract of animals and man, these Gram-positive bacteria form spores which are resistant to heating compared with vegetative cells. *C. perfringens* in the intestine exists both as spores and vegetative cells, spores are also found in environmental samples. The spores of *C. perfringens* survive in water for months, much longer than vegetative faecal indicator bacteria and consequently their presence may indicate remote or intermittent faecal pollution. 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 the treatment-works performance. The spores are not always inactivated by routine disinfection procedures (e.g. chlorination).

INTERNATIONAL STANDARD ISO 14189:2013(E)

Water quality — Enumeration of *Clostridium perfringens* — Method using membrane filtration

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 and to ensure compliance with any national regulatory conditions.

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

1 Scope

This International Standard specifies a method for the enumeration of vegetative cells and spores of *Clostridium perfringens* by the membrane filtration method in samples of water intended for human consumption. However, the method can be applied to all types of water samples provided they do not contain particulate or colloidal matter that interferes with filtration.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 guidance on the enumeration of micro-organisms by culture*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

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

ISO/IEC Guide 2:2004, *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:

3.1
presumptive *Clostridium perfringens*
bacteria which produce all shades of black or grey to yellow brown colonies on tryptose-sulfite-cycloserine agar, even if the colour is faint, after anaerobic incubation at (44 ± 1) °C for (21 ± 3) h

Note 1 to entry: Unlike colonies growing directly on the agar medium, colonies on the membrane do not always display a distinct blackening, so faint colonies are included in the count.

3.2
confirmed *Clostridium perfringens*
bacteria that produce characteristic colonies on tryptose-sulfite-cycloserine agar and possess the enzyme acid phosphatase

4 Principle

A measured volume of the sample, or a dilution of it, is filtered through a membrane with a pore size of 0,45 µm sufficient to retain spores of clostridia. The membrane is incubated on a selective/differential agar (tryptose-sulfite-cycloserine agar) anaerobically at (44 ± 1) °C for (21 ± 3) h. *C. perfringens* usually produce black or grey to yellow brown colonies as a result of the reduction of sulfite to sulfide which reacts with a ferric salt in the medium. Characteristic colonies are counted and confirmatory tests are carried out. The result is calculated as the colony count per sample volume. If a count of spores alone is required the sample is first pre-treated at (60 ± 2) °C to inactivate vegetative bacteria.

NOTE 1 The medium contains cycloserine as a selective agent to inhibit *Bacillus* species.

NOTE 2 Incubation at 44 °C increases the selectivity of the test for *C. perfringens.*

5 Apparatus and glassware

Except for disposable glassware or plastics ware which is delivered sterile, sterilize glassware as specified in ISO 8199.

Usual microbiological equipment and particularly:

5.1 Membrane filtration apparatus, as specified in ISO 8199.

5.2 Sterile filter funnels

Use funnels either delivered sterile or sterilized as specified in ISO 8199. Alternatively flaming of funnels made of metal prior to their use is acceptable.

NOTE For this method it is insufficient to disinfect funnels by boiling.

5.3 Sterile membrane filters, nominal pore size 0,45 µm.

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

5.4 Incubators, capable of being maintained at (36 ± 2) °C and at (44 ± 1) °C.

5.5 Water bath (optional), capable of being maintained at (60 ± 2) °C equipped with a means of circulating the water.

5.6 Autoclave, capable of being maintained at (121 ± 3) °C.

5.7 Sterile forceps

5.8 Anaerobic jars, or similar equipment.

5.9 Anaerobic gas generating system, to generate an atmosphere of approximately 90 % hydrogen and 10 % carbon dioxide.

6 Culture media and reagents

6.1 Basic materials

For uniformity of results, in the preparation of media, use constituents of uniform quality and chemicals of recognized analytical grade. For preparation of the media use glass-distilled water or deionized water of equivalent purity, as specified for water grade 3 in ISO 3696[1].

Alternatively, use commercially available dehydrated complete medium and reagents prepared and used according to the manufacturer's instructions.

Other grades of chemicals may be used provided they can be shown to lead to the same results.

6.2 Culture media

See Annex A.

6.2.1 Tryptose sulfite cycloserine agar (TSC agar), References [6][11][12]

See A.1.

6.2.2 Blood agar or Columbia agar base or another suitable nutrient-rich agar

See A.2.

6.2.3 Acid phosphatase reagent

See A.3.

7 Sampling

Carry out sampling as specified in ISO 19458.

8 Procedure

8.1 Transport and storage of the sample

Start examination as soon as possible after the collection of the sample preferably within the same working day. Samples should be cooled during transport ideally at (5 ± 3) °C. The recommended maximum sample storage time including transport is for vegetative bacteria 12 h and for spores 24 h. The sample storage time including transport shall not exceed 18 h for vegetative bacteria and 72 h for spores.

8.2 Heat pre-treatment to select spores

If it is the intention to count only spores, mix the sample well and then heat it to (60 ± 2) °C in a water bath 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 ± 2) °C shall not exceed 15 min and can be minimized by ensuring the water in the water bath is circulated to maximize heat exchange.

8.3 Sample dilution

A test volume of sample or dilution of it - after heat treatment if required - should be chosen to yield, if possible, between 10 and 80 colonies on a membrane 47 mm to 50 mm in diameter. Test volumes or dilutions should be prepared as described in ISO 8199.

8.4 Filtration

For a general description of the membrane filtration technique see ISO 8199.

Filter a measured volume of water. The volume should be appropriate to the water being examined. For water intended for human consumption, it is usual to filter a volume of 100 ml. Record the volume filtered.

After filtration, place the membrane grid face upwards on a TSC agar plate ensuring that no air bubbles are trapped under the filter.

NOTE Alternatively, a thin layer (about 5 ml to 10 ml related to a petri dish of 90 mm diameter) molten TSC agar (equilibrated in a water bath at (45 ± 1) °C) as an overlay on the filter can be used. Allow to solidify before anaerobic incubation. This procedure may enhance the blackening of the colonies. It is not necessary to add cycloserine in the TSC agar for the overlay. However, obtaining pure cultures for the confirmation test may be more laborious.

As the spores of *C. perfringens* are more heat resistant, sterile funnels shall be used (5.2). Placing in a boiling water bath between samples may not be sufficient to inactivate spores. Flaming of metal funnels is considered acceptable. For different volumes of the same sample, the funnel may be reused provided the smallest volumes of sample are filtered first.

8.5 Incubation and examination

The time between placing the membrane on the TSC agar and starting incubation should be as short as possible and shall not exceed 1 h.

Incubate the plates with the filters, anaerobically at (44 ± 1) °C for (21 ± 3) h inverted to avoid interference with condensing water.

After incubation, enumerate the presumptive *C. perfringens* by counting all colonies which show black or grey to yellow brown staining, even if the colour is faint, of the TSC medium when viewed from either above or below the membrane filter.

Since the black colour of the colonies rapidly fades and finally disappear, the plates have to be counted within 30 min after completion of the anaerobic incubation. If more anaerobic jars are used, the plates should be checked jar by jar or in portions if the incubation was performed in an anaerobic incubator.

8.6 Confirmation of *Clostridium perfringens*

8.6.1 General

It is recommended for membrane filtration methods for water analysis that for counts of 1 to 10 all colonies are subject to confirmation, and for counts above 10, at least 10 colonies taken at random are subject to confirmation.

For confirmation subculture all colonies for counts of 1 to 10 and at least 10 colonies for counts above 10 taken randomly onto blood agar plates. When this is impracticable, all typical colonies shall be examined from a sub-area of the membrane.

If blood agar is not available, Columbia agar base or another nutrient-rich agar (e.g. tryptone soya agar) may be used for subculture.

Incubate anaerobically in an incubator at (36 ± 2) °C for (21 ± 3) h.

8.6.2 Acid phosphatase test

Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent are placed onto the colonies. A purplish colour developed within 3 min to 4 min is considered as a positive reaction.

8.6.3 Interpretation

C. perfringens produces black or grey to yellow brown colonies on TSC agar, even if the colour is faint, and possesses acid phosphatase.

9 Expression of results

From the numbers of total and confirmed colonies, calculate the numbers of presumptive *C. perfringens* and *C. perfringens* and number or spores, if applicable, present in 100 ml of the filtered volume in accordance with ISO 8199.

Where required, the variability of the test results should be evaluated according to ISO 29201[3].

10 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (ISO 14189:2013);

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

c) the number of colonies presumptive *C. perfringens* (optional, depending on the purpose of the investigation);

d) the number of colonies confirmed as *C. perfringens*;

e) information, if the result represents the total number of *C. perfringens* (vegetative cells and spores) or spores only;

f) any particular occurrence(s) observed during the analysis and any operation(s) not specified in this method, which may have affected the results.

11 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.

Media quality control is described in ISO/TS 11133-1. For quantitative process quality control a suspension of *C. perfringens* is used for this purpose. Select the volume filtered to contain between 10 cfu to 80 cfu and treat the control like a sample. Compare recovery to that on a non-selective agar such as blood agar. Alternatively to the suspension of *C. perfringens* use reference materials.

NOTE Since the medium should be used as fresh as possible it is acceptable to perform media and process control for samples simultaneously in parallel with the analysis of the samples.

Include a blank control in each batch of analyses. Filter 100 ml of sterile water or another appropriate diluent according to ISO 8199 and continue to treat it like a sample but without pasteurization. No colonies should be visible after incubation.

Include an appropriate control for correct anaerobic conditions (e.g. anaerobic indicator strip) whenever anaerobic incubation is performed (anaerobic jar or anaerobic incubator).

For the confirmation step performed by acid phosphatase test include known positive and negative control strains.

At least one of the *C. perfringens* strains given in Table 1 shall be used as a positive control for media control and the confirmation test.

Bacillus subtilis WDCM 00003 shall be used as a negative control for assessing TSC medium and anaerobic conditions.

Clostridium bifermentans WDCM 00079 shall be used as negative control for the confirmation test.

Table 1 — Performance testing of Tryptose sulfite cycloserine agar (TSC agar) by comparing with a non-selective reference medium

Function	Incubation	Control strains[a]	Reference media	Method of control	Criteria	Characteristic reactions
Productivity	(21 ± 3) h / (44 ± 1) °C anaerobic	*Clostridium perfringens* WDCM 00007; *C. perfringens* WDCM 00080; *C. perfringens* WDCM 00174	TSA or blood agar	Quantitative	*PR* ≥ 0,5	Black colonies
Selectivity	(21 ± 3) h / (44 ± 1) °C anaerobic	*Bacillus subtilis* WDCM 00003	—	Qualitative	Total inhibition (0)	—

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

Annex A
(normative)

Composition and preparation of culture media and reagents

A.1 Tryptose sulfite cycloserine agar (TSC agar), References[6][11][12]

A.1.1 Basal medium

Enzymatic digest of casein	15 g
Enzymatic digest of soya	5 g
Yeast extract	5 g
Sodium disulfite (sodium metabisulfite), anhydrous ($Na_2S_2O_5$) (CAS No 7681-57-4)	1 g
Iron(III) ammonium citrate (CAS No.: 1185-57-5)	1 g
Agar	9 g to 18 g
Water	1 000 ml

Suspend the ingredients in the water and dissolve by heating and stirring. Sterilize by autoclaving at (121 ± 3) °C for 15 min. Allow to cool to 45 °C to 50 °C. The basal medium may be stored at (5 ± 3) °C and used within 4 weeks of preparation. To prepare the complete medium the stored medium is remelted by steaming and cooled to 45 °C to 50 °C before adding the cycloserine solution (see A.1.2).

A.1.2 D-cycloserine solution

D-cycloserine (CAS No.: 68-41-7)	4 g
Water to	100 ml

Dissolve the D-cycloserine in the water and filter through a membrane of 0,2 µm pore size. Dispense aseptically into suitable volumes, store at (-20 ± 5) °C and use within 4 weeks of preparation. Alternatively, the dispended volumes of cycloserine can be stored at (-70 ± 10) °C for a maximum of 12 months.

A.1.3 Complete medium

Basal medium (A.1.1)	1 000 ml
D-cycloserine solution (A.1.2)	10 ml

Add the D-cycloserine solution to the molten cooled basal medium, mix well and dispense into vented Petri dishes to a depth of at least 5 mm.

The final pH of the medium should correspond to 7,6 ± 0,2 at 25 °C.

Use the prepared plates as fresh as possible on the same day. If storage of the prepared plates is inevitable, store the plates under anaerobic conditions at (5 ± 3) °C and use them within 7 d.

Discard plates, once removed from the refrigerator, if not used. Do not return the plates to storage, as the performance of the medium deteriorates. Dry the plates well before use.

A.2 Blood agar

Columbia blood agar or another suitable base with 5 % blood (e.g. horse, sheep blood).

If blood agar is not available another suitable non selective nutrient rich agar may be used for subculture (e.g. Columbia agar base or tryptone soya agar).

A.3 Acid phosphatase reagent

1-naphthylphosphate disodium salt (CAS No.: 2183-17-7)	0,4 g
Fast Blue B Salt (o-Dianisidine bis(diazotized) zinc double salt) (CAS No 14263-94-6)	0,8 g
Acetate buffer (pH 4,6 ± 0,2)	20 ml

Prepare the acetate buffer by dissolving 0,3 ml glacial acetic acid (CAS No.: 64-19-7) and 0,4 g sodium acetate (CAS No.: 127-09-3) in deionized water and make up to 1 000 ml. Alternatively use a commercially available product.

Dissolve the ingredients in the acetate buffer and allow to stand for (60 ± 5) min at (5 ± 3) °C to allow precipitation. Then filter the solution through a fluted filter to remove the precipitate. Store the prepared solution at (5 ± 3) °C for no longer than two weeks. If precipitation occurs again filter again once more before use.

NOTE 1 Instead of 1-naphthylphosphate disodium salt 1-naphthylphosphate monosodium salt (CAS No.: 81012-89-7) can be used.

CAUTION — Fast Blue B Salt is toxic and may cause cancer – appropriate precautions shall be taken when weighing out, preparing and discarding the reagent.

Annex B
(informative)

Performance data

B.1 Performance characteristics of the standard method

In an intralaboratory trial the performance parameters like range of quantitative determination, robustness of incubation time, counting uncertainty and efficiency, have been determined, References. [2][10] The data are summarized in Table B.1.

A collaborative study within 12 laboratories from 11 countries was performed (Austria, Czech Republic, Finland, France, Germany, Hungary, Ireland, Portugal, Slovakia, The Netherlands, United Kingdom) to investigate the precision of the method. The samples (n = 108) consisted of reference material (lenticules, mixed culture, kindly provided by HPA, UK). The reference material was delivered to the participants and used to prepare the water samples. On three days within a time span of two week analyses of three samples in triplicate each were performed according to the standard method. One plate per sample and day of analyses was used for confirmation; all colonies of these plates were tested with acid phosphatase-test. The recovery of the method was determined by using blood agar plates for cultivation. The data are presented in Tables B.1 and B.2.

Table B.1 — Parameters for performance characteristics

Parameter	
Intralaboratory trial	
Range of quantitative determination (colonies per membrane 47 mm in diameter)	10 to 80
Robustness of incubation time	18 h to 24 h no significant difference in counts
Counting uncertainty (RSD)	
repeatability	0,038
reproducibility (intralab)	0,0348
Identification (n=127)	
sensitivity (%)	94
specificity (%)	87
false positive rate	0,10
false negative rate	0,09
selectivity	-0,15
efficiency (%)	92
Interlaboratory trial	
Precision (RSD)[a]	
repeatability (within lab)	0,1676
reproducibility within lab	0,1382
reproducibility (between lab)	0,3232
Recovery (%)	>72

[a] Both, distribution variability and operational variability are included in these assessments.

B.2 Statistical procedure used for the determination of the precision parameters of the collaborative study – Nested random effects in data analysis: Two-way ANOVA

The main principle was to fit data to a linear model (additive effect of the error, without interaction). The counts were transformed into lg scale in order to comply with the Normality pre-requisites of ANOVA. The Grubbs Test was used to detect outlier (suspicious result for one laboratory - *n°6*). No result was discarded.

The model described in ISO Guide 35[4] was used. Regarding the statistical pattern of the collaborative study, the original 'between-bottle' variance was changed into a 'between-day' variance.

$$x_{kij} = \mu + \alpha_k + \beta_{ki} + \varepsilon_{kij}$$

where

x_{kij} is the measurement j of day i for the laboratory k;

μ is the true value;

α_k is the error due to the laboratory k;

β_{ki} is the error due on i^{th} day within the laboratory k;

ε_{kij} is the random error of measurement.

From a practical point of view:

— m is the estimation of μ (consensus value also called assigned value or grand mean);

— S_L^2 is the variance due to the interlaboratory error α (systematic error);

— S_u^2 is the variance due to the "day effect" β;

— S_r^2 is the variance of the measurement error ε.

NOTE The variance of reproducibility can be assessed as follows: $s_R^2 = s_L^2 + s_r^2$.

All these parameters were estimated simultaneously by the method of analysis variance (ANOVA). The same number of repeated measurements for each day and the same number of days per laboratory were considered.

Finally, the formula given in ISO 29201:2012, 6.2 used to convert lg scale into natural logarithms was applied to the estimated variance. This conversion leads to an expression of the uncertainty in a relative scale (relative variance: see also ISO 29201:2012, 2.5.2):

$$u_R^2 = 5{,}3019 s_R^2$$

$$u_u^2 = 5{,}3019 s_u^2$$

$$u_r^2 = 5{,}3019 s_r^2$$

Table B.2 — Collaborative study: Results of the determination of the precision parameters

	Var (lg scale)	u^2	u
Interlaboratory error	0,0144	0,0763	27,6%
"day-effect"	0,0036	0,0190	13,8%
Measurement error	0,0053	0,0279	16,7%
Reproducibility (between lab)	0,0197	0,1043	32,3%

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