
**Water quality — Detection and
enumeration of thermotolerant
Campylobacter spp**

*Qualité de l'eau — Recherche et dénombrement d'espèces
thermotolérantes du genre *Campylobacter**

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	2
4.1 General	2
4.2 Inoculation and enrichment in selective liquid medium	2
4.3 Isolation and selection for confirmation	2
4.4 Confirmation	2
5 Culture media and reagents	3
6 Apparatus	3
7 Sampling, transport and storage	4
8 Sample processing	4
8.1 General	4
8.2 Direct inoculation into enrichment broth	4
8.3 Membrane filtration	4
9 Procedure	5
9.1 Enrichment	5
9.2 Plating on solid, selective medium	6
9.3 Reading of presumptive results	6
9.4 Confirmation	6
9.4.1 General	6
9.4.2 Selection of colonies for confirmation	6
9.4.3 Absence of growth on non-selective agar plates	7
9.4.4 Motility and cell morphology	7
9.4.5 Detection of oxidase activity	7
9.4.6 Interpretation	7
9.4.7 Further verification	8
9.5 Identification of <i>Campylobacter</i> species (optional)	8
9.5.1 General	8
9.5.2 Detection of catalase	8
9.5.3 Detection of hippurate hydrolysis	8
9.5.4 Detection of indoxyl acetate hydrolysis	9
9.5.5 Interpretation	9
10 Quality assurance	9
11 Expression of results	9
12 Test report	10
Annex A (normative) Flow diagram of the method	11
Annex B (normative) Semi-quantitative analysis	12
Annex C (normative) Composition and preparation of culture media and reagents	13
Annex D (normative) Performance testing for the quality assurance of the culture media	22
Annex E (informative) Performance characteristics	23
Annex F (informative) Additional information about campylobacters	24
Bibliography	25

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 17995:2005), which has been technically revised. The main changes compared to the previous edition are as follows:

- the inclusion of direct inoculation of enrichment broths in addition to membrane filtration with additional information about sample processing.
- methods for the speciation of *Campylobacter*.
- performance testing for the quality assurance of culture media has been added to [Annex D](#).
- performance characteristics of the method have been added as an [Annex E](#).

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.

Introduction

The thermotolerant *Campylobacter* species are not thought to propagate as free living but are zoonotic pathogenic bacteria of mammals and birds and which cause disease in humans. *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli* are common causes of intestinal infections in humans. *Campylobacter upsaliensis* is found mainly in cats and dogs and is of minor importance for human infections. *Campylobacter lari* is less frequently associated with human infections. *Campylobacter* infections give rise to a flu-like illness with malaise, fever and myalgia followed by diarrhoea. The vehicles for *Campylobacter* infections are usually food, farm animals, pets and person-to-person contact; water is also important. They can be isolated from waters contaminated with human or animal faeces such as wastewater and surface waters. The bacteria have been demonstrated to survive within amoebae. Outbreaks of campylobacteriosis have been reported in relation to the use of contaminated drinking water and sporadic cases from recreational water use.

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Water quality — Detection and enumeration of thermotolerant *Campylobacter* spp

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 be carried out by suitably trained staff.

1 Scope

This document specifies a method for the detection, semi-quantitative and quantitative (MPN) enumeration of thermotolerant *Campylobacter* species.

The method can be applied to all kinds of waters including: drinking water, ground water and well water, fresh, brackish and saline surface water, swimming pools, spa and hydrotherapy pools, recreational waters, agricultural waters and runoff, untreated and treated wastewater and also sand and other sediments.

This method can be used for the detection of *Campylobacter* species in a specified sample volume. Clean water samples with low turbidity can be membrane filtered for either a qualitative method, semi-quantitative or quantitative (MPN) method. Water samples with higher turbidity, such as primary and secondary wastewater effluents and sediments, are analysed using the same qualitative, semi-quantitative or quantitative MPN method by direct inoculation of material into bottles or tubes. Sediments can be suspended in a suitable diluent or inoculated directly into enrichment broths.

Users wishing to employ this method are expected to verify its performance for the particular matrix under their own laboratory conditions.

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 requirements and guidance for microbiological examinations by culture*

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

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

3 Terms and definitions

For the purposes of this document, the following terms and definitions 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

Campylobacter

microorganisms forming characteristic colonies on solid selective media when incubated in a microaerobic atmosphere at 41,5 °C and which possess the characteristic morphology, motility, biochemical and growth properties described when tests are conducted in accordance with ISO 17995

Note 1 to entry: Thermotolerant *Campylobacter* species of relevance in human infections include *Campylobacter jejuni* subsp. *jejuni* (hereafter referred to as *Campylobacter jejuni*), *Campylobacter coli*, *Campylobacter lari* and possibly *Campylobacter upsaliensis*. *Campylobacter upsaliensis* is found mainly in cats and dogs and is of minor importance for human infection.

3.2

detection of *Campylobacter*

determination of the presence or absence, semi-quantitative enumeration or quantitative enumeration using a most probable number (MPN) system of *Campylobacter* in water and solid materials when the test is conducted in accordance with ISO 17995

4 Principle

4.1 General

In general, the detection of *Campylobacter* requires enrichment followed by isolation of colonies and their confirmation. The flow diagram of the procedure in [Annex A](#) shall be applied.

4.2 Inoculation and enrichment in selective liquid medium

Samples are inoculated either directly or after concentration using membrane filtration into one of two selective enrichment broths depending on the expected level of background microorganisms: Bolton broth for clean water and Preston broth for more heavily contaminated water. A single sample volume is processed for *Campylobacter* detection and, where necessary, at least three 10-fold volumes (for example 10 ml, 100 ml and 1 000 ml) are used for a semi-quantitative determination ([Annex B](#) shall be applied). For a quantitative (MPN) determination, volumes of 500 ml, 5 × 100 ml, 5 × 10 ml and, where counts may be high, smaller volumes are used or the initial sample is diluted. The broths are then incubated microaerobically at $(37 \pm 1) ^\circ\text{C}$ for (44 ± 4) h.

The ratio of sample to enrichment broth shall be 10 % or less if single strength broth is used.

4.3 Isolation and selection for confirmation

From the enrichment broth cultures in [4.2](#), liquid selective media are inoculated onto modified charcoal cefoperazone deoxycholate agar (mCCDA).

The mCCDA plates are then incubated at $(41,5 \pm 1) ^\circ\text{C}$ for (44 ± 4) h in a microaerobic atmosphere and examined for characteristic colonies after incubation. Colonies with typical characteristics are presumed to be *Campylobacter* (see [9.3](#)). It is necessary to confirm at least one colony from each plate to demonstrate that the corresponding enrichment culture is positive for the presence of *Campylobacter* species.

4.4 Confirmation

Confirmation of suspect colonies of *Campylobacter* species involves biochemical, morphological and physiological tests. See flow diagram in [Annex A](#).

The colonies presumed to be *Campylobacter* are inoculated on the non-selective Columbia agar or other appropriate solid non-selective media, and are then confirmed by means of microscopic examination, failure to grow aerobically at 25 °C and appropriate biochemical tests. Optionally, *Campylobacter* species are further identified by specific biochemical tests.

For species identification, it is recommended that more than one isolate per enrichment culture is tested.

If typical *Campylobacter* species are confirmed or identified, the result is given as the qualitative, semi-quantitative or quantitative (MPN) estimate per volume of sample.

5 Culture media and reagents

For current laboratory practice see ISO 8199 and ISO 11133.

The composition and preparation of the culture media and reagents given in [Annex C](#) shall be used. The performance testing requirements given in [Annex D](#) shall be applied.

6 Apparatus

Usual microbiological laboratory equipment (as specified in ISO 8199) and, in particular, the following:

- 6.1 Water baths or incubators**, capable of operating at $(37 \pm 1) ^\circ\text{C}$ and $(41,5 \pm 1) ^\circ\text{C}$.
- 6.2 Water bath**, capable of operating between $47 ^\circ\text{C}$ and $50 ^\circ\text{C}$.
- 6.3 Membrane filtration equipment**, as specified in ISO 8199.
- 6.4 Membrane filters**, sterile membrane filters made of cellulose ester with a diameter of 45 mm to 50 mm and a pore size of $0,45 \mu\text{m}$. Larger diameter membranes may be used with suitable filter holders provided they have a pore size of $0,45 \mu\text{m}$.
- 6.5 pH-meter**, accurate to within 0,2 pH units at $25 ^\circ\text{C}$.
- 6.6 Apparatus suitable for achieving a microaerobic atmosphere**, with oxygen content of $(5 \pm 2) \%$, carbon dioxide $(10 \pm 3) \%$, optional hydrogen at $\leq 10 \%$, with the balance being nitrogen.

Appropriate gastight containers of, for example, 3,5 l capacity are used to hold Petri dishes and/or flasks or bottles for the enrichment broth, such as bacteriological anaerobic jars. The appropriate microaerobic atmosphere can be obtained using commercially available gas-generating kits, following precisely the manufacturer's instructions, particularly those relating to the volume of the jar and the capacity of the gas-generating kit. The jar may be flushed and filled with an appropriate gas mixture prior to incubation. Alternatively, an incubator with appropriately modified atmosphere (approximately 5 % oxygen and 10 % carbon dioxide) can also be used.

Gas-generating pouches can be used if they are able to maintain an atmosphere with approximately 5 % oxygen and approximately 10 % carbon dioxide.
- 6.7 Microscope**, preferably with phase contrast or differential interference contrast. Dark ground illumination can also be used.
- 6.8 Bottles**, 100 ml to 250 ml, with screw caps for the selective enrichments.
- 6.9 Vented Petri dishes**, sterile, 90 mm.
- 6.10 Disinfected forceps**, for handling membrane filters.
- 6.11 Refrigerator**, capable of operating at $(5 \pm 3) ^\circ\text{C}$.

7 Sampling, transport and storage

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Be aware that campylobacters are very sensitive to adverse conditions. Keep samples cool (5 ± 3) °C and in the dark until they are processed. The temperature should not be above that at which the sample was taken, nor should the sample be allowed to freeze. Avoid unnecessary mixing with air. Process the samples as soon as possible after collection, but within a maximum of 30 h. In all other aspects of sampling, transport and storage, follow the instructions given in ISO 19458.

NOTE 1 Campylobacters survive well in clean water at (3 ± 2) °C. At higher temperatures or in other media, they can quickly deteriorate.

NOTE 2 It is important that sample bottles are filled to the rim. This avoids air spaces in the container and helps to ensure *Campylobacter* survival.

8 Sample processing

8.1 General

Depending on the sample properties and expected level of faecal contamination, use direct inoculation (see 8.2) or membrane filtration (see 8.3) for sample processing. Direct inoculation is suitable for samples with a high contamination level such as wastewater, whereas membrane filtration is suitable for surface water, groundwater and drinking water samples. Direct inoculation is only suitable for relatively small volumes, for example 100 ml or less and membrane filtration is suitable for larger volumes.

The ratio of a maximum of 10% of sample to enrichment broth is used (for example 100 ml of sample to 1 L of broth) to avoid significant change to the composition of the medium. In addition, background microorganisms present in the sample are diluted sufficiently to avoid their inhibition of the growth of campylobacters during enrichment.

Throughout the processing steps, care should be taken to minimise the risk of cross-contamination of enrichment broths. The use of pipette tips with sterile filters is essential if automated pipettes are used. In addition any positive controls should be processed separately.

8.2 Direct inoculation into enrichment broth

Samples with expected high contamination levels are inoculated directly into Preston broth (see C.2). Where the expected level of background microorganisms is low and samples cannot be processed by membrane filtration, Bolton broth (see C.3) may be used. If no information about the contamination level is available, both broths should be used.

Double strength broth should be used for large volumes. Single strength broth should be used for relatively small volumes of water where the ratio of sample to enrichment broth shall be 10 % or less. Generally, volumes of 100 ml, 50 ml and 10 ml are inoculated into an equal volume of double strength broth. Volumes of 1 ml and 0,1 ml if required are inoculated into 10 ml of single strength broth.

Solid material can be weighed directly into single strength enrichment broths. Aliquots of 10 g can be weighed into 100 ml of enrichment broth, 1 g and smaller aliquots should be weighed into 10 ml of enrichment broth.

NOTE When high numbers of campylobacters are expected, the sample can also additionally be streaked directly onto mCCDA (see C.4) without prior selective enrichment.

8.3 Membrane filtration

Samples with expected low levels of contamination are filtered using membrane filters with a pore size of 0,45 µm (see 6.4). Select one of the two enrichment broths depending on the expected level of background

microorganisms and use 100 ml of single strength broth for the enrichment of each sample volume. Bolton broth (see [C.3](#)) is used for clean samples and Preston broth (see [C.2](#)) for heavily contaminated water. If no information about the contamination level is available, both broths should be used.

For detection of campylobacters in drinking water, other treated waters and clean surface waters, a 1 000 ml sample is filtered. If required, larger volumes may be processed using more filters or, where necessary, larger diameter filters may be used. Alternative concentration methods may be used, for example ultra-filtration or centrifugation providing that they are verified for recovery by the user. For more contaminated water, smaller volumes should be used and, for samples containing more particulate material, several membrane filters may be used to filter the required volume. All membrane filters from one sample volume shall be placed in the same container of enrichment broth.

For a semi-quantitative determination, volumes of 10 ml, 100 ml and 1 000 ml are filtered (see [Annex B](#)). The smaller volumes of 10 ml can also be pipetted directly into 100 ml of single strength medium or 10 ml of double strength medium.

For quantitative estimates, an MPN series can be used. In an MPN series, 100 ml and larger volumes can be filtered and membrane filters from different volumes are placed into 100 ml of separate single strength broth. Smaller volumes of 10 ml can be pipetted into 100 ml of single strength medium or 10 ml of double strength medium, and 1 ml can be pipetted into 10 ml of single strength medium. For example, volumes of 5 × 100 ml and 500 ml can be filtered and membranes placed into 100 ml of single strength broth. Volumes of 10 ml can be directly inoculated into 5 × 100 ml of single strength medium or 5 × 10 ml of double strength medium. Volumes of 5 × 1 ml can be inoculated directly into 10 ml of single strength broth.

After filtration, the membranes are transferred using disinfected forceps (see [6.10](#)) into enrichment broths.

9 Procedure

9.1 Enrichment

For samples known to have low concentrations of background microorganisms, use the less selective Bolton broth for enrichment and for samples expected to have a high concentration of background microorganisms use the highly selective Preston broth. For samples where the background microbial concentration is unknown, enrich parts of each sample in both Preston and Bolton broths. Preston broth may be too selective to allow the recovery of some strains of *C. coli*. Bolton broth may not be selective enough to counteract the growth of non-campylobacters in some samples. If the available sample size is limited, choose one of the enrichment broths which is considered to be the most appropriate.

NOTE 1 The amount of sample (volume or weight) to be analysed can vary with the sample material and the scope of the investigation.

Bring enrichment broths to room temperature prior to inoculation.

Transfer the samples (see [8.2](#) and [8.3](#)) to bottles with the appropriate volumes of enrichment broth(s). Put the inoculated broths in jars ([6.6](#)) or in an incubator with modified atmosphere. Leave the caps loosely placed on the inoculated broths during incubation to allow the modified atmosphere to reach the broths. Apply the modified atmosphere to the jars ([6.6](#)) and incubate at $(37 \pm 1)^\circ\text{C}$ for (44 ± 4) h.

NOTE 2 The Preston campylobacter-selective supplement (see [C.2.2](#)) contains antibiotics (polymyxin B and rifampicin) known to be rather toxic towards *C. coli* and towards sub-lethally injured *C. jejuni*. Accordingly, pre-enrichment for 4 h in Preston broth without the selective supplement prior to the enrichment in the complete Preston broth (see [C.2](#)) has been found by some laboratories to increase the recovery of campylobacters from waters with low numbers of other microorganisms. The Bolton broth selective supplement (see [C.3.2](#)) does not include antibiotics known to be toxic towards campylobacters.

NOTE 3 Some campylobacters can die or grow too slowly if the incubation temperature is below 36°C .

9.2 Plating on solid, selective medium

After incubation for (44 ± 4) h, remove the broths carefully from the jars to avoid re-suspension of sedimented material including background microorganisms. With a sterile loop, transfer approximately 10 µl of enrichment culture onto the surface of an mCCDA (C.4) plate. Draw the inocula from just below the surface of the broths and streak onto the surface of the plates. Without delay, incubate the inoculated plates in jars with a modified atmosphere (see 6.6) at $(41,5 \pm 1)$ °C for (44 ± 4) h.

NOTE 1 Bolton broth (see C.3) contains cefoperazone. The use of a second plating medium with selective agents different from those in mCCDA (see C.4) could improve *Campylobacter* detection, especially in the presence of background microflora resistant to third generation β-lactams like cefoperazone.

NOTE 2 The use of additional mCCDA plates for spread-plating 100 µl of enrichment broths can enhance the detection of low counts or injured *Campylobacter*.

9.3 Reading of presumptive results

Examine the inoculated mCCDA plates for visible growth after incubation.

Typical colonies of campylobacters are small, flat or convex with a greyish glossy surface often with a metallic sheen. They have a tendency to spread along the inoculation tracks. Well-spaced colonies resemble droplets of fluid. On moist agar, a thin, spreading film may be seen. Colony mass collected on a loop has a tan or creamy colour. See Annex F for additional guidance. Images of *Campylobacter* colonies on mCCDA are available at <https://standards.iso.org/iso/17995/ed-2/en>.

In cases of doubt, collect colony material from the surface of the plates and check under the microscope for typical appearance (see 9.4.4).

With continued incubation, colonies become flat or convex with a dull surface. A metallic sheen may develop. The colour of the colonies varies from transparent to greyish or whitish.

NOTE After the first examination of plates, the prolonged incubation of negative plates for up to 92 h might enhance the detection of injured *Campylobacter*, for example, from disinfected waters.

9.4 Confirmation

9.4.1 General

Throughout all tests, be aware that cultures may deteriorate quickly in light and air and follow the procedures in 9.4 without delay. At least one colony from each plate needs to be confirmed to say the corresponding enrichment broth was positive.

If speciation is required, several colonies should be subcultured and, if necessary, the cultures sent to an appropriate reference laboratory.

Alternative methods for confirmation and species identification such as commercially available biochemical test kits, gene amplification using genus and species specific PCR assays or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) may be used provided they have been verified by the user.

9.4.2 Selection of colonies for confirmation

For confirmation, take from each plate of mCCDA (see 9.3) at least one colony considered to be typical or suspected of being *Campylobacter*. One confirmed isolate per sample is sufficient. If the first colony is negative, select up to four more colonies.

If needed, store the original isolation plates (see 9.3) under microaerobic conditions at (5 ± 3) °C for use in further confirmation and /or identification. *Campylobacter* isolates can lose their culturability when stored aerobically at 5 °C.

Streak each of the suspected colonies onto a blood agar plate (see [C.5](#)), mCCDA basal medium without supplements (see [C.4.1](#)), Columbia agar (see [C.5](#)), or other non-selective agar plates which have been demonstrated to support the growth of campylobacters, in order to allow the development of well-isolated colonies. Incubate the plates in a microaerobic atmosphere at 41,5 °C from (24 ± 2) h to (44 ± 4) h. Use well-isolated freshly grown colonies for the examination of growth aerobically at 25 °C (see [9.4.3](#)), morphology and motility (see [9.4.4](#)), oxidase (see [9.4.5](#)) and also for identification (see [9.5](#)).

9.4.3 Absence of growth on non-selective agar plates

Using the cultures obtained in [9.4.2](#), inoculate a rich, non-selective agar plate, blood agar (see [C.5](#)), mCCDA basal medium without supplements (see [C.4.1](#)), Columbia agar (see [C.5](#)), or other non-selective agar plates which have been demonstrated to support the growth of campylobacters. Incubate the plates aerobically at 25 °C for (44 ± 4) h.

Campylobacters do not grow aerobically at 25 °C. *Arcobacter* spp. will grow at 25 °C aerobically and resemble *Campylobacter* in colony appearance, morphology and motility, and reaction to the oxidase test.

Positive and negative controls should be analysed alongside to confirm the results. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control, no growth) and *Escherichia coli* WDCM 00012 (negative control, good growth).

9.4.4 Motility and cell morphology

Suspend material from a suspect colony in a suitable nutrient broth like Preston basal broth (see [C.2.1](#)) on a slide. Cover with a cover slip and examine immediately under the microscope, preferably using phase contrast or differential interference contrast. Alternatively, motility may be demonstrated by similar microscopy using a hanging drop (see Reference [1]).

Campylobacters are highly motile, slender rods with a spiral appearance. Motility is characterized by darting or corkscrew-like movements. Campylobacters can be immobilized prior to microscopy for typical morphology by suspending in water instead of a nutrient broth. As an alternative to phase contrast microscopy techniques for examining the appearance, campylobacters can be studied by dark-field microscopy or after staining a smear for 5 min with 20 g/l carbol-fuchsin. Gram staining often gives unsatisfactory results with campylobacters.

Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Escherichia coli* WDCM 00012 (negative control).

9.4.5 Detection of oxidase activity

Perform the test using fresh colony material from a non-selective agar (see [C.5](#) or [C.4.1](#)) and oxidase reagent (see [C.6](#)) on a filter paper in a Petri dish. With a platinum (not Ni chrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth onto the prepared filter paper. Regard the appearance of a deep blue-purple colour within 10 s as a positive reaction. Commercial oxidase strips should be read in accordance with the manufacturer's instructions.

Campylobacters are oxidase-positive, but the reaction may be delayed by up to 10 s. Positive and negative controls should be analysed alongside to confirm the results. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (oxidase positive control) and *Escherichia coli* WDCM 00012 (oxidase negative control).

9.4.6 Interpretation

Campylobacter gives results in accordance with [Table 1](#). If typical campylobacters are present, report this as indicated in [Clause 11](#).

Table 1 — Characteristics of *Campylobacter*

Aerobic growth (9.4.3)	–
Motility (9.4.4)	Characteristic darting, often rotating, motility
Morphology (9.4.4)	Curved or spiral shaped rods ^a
Oxidase (9.4.5)	+
+ = positive.	
– = negative.	
^a Older cultures may rapidly lose their characteristic shape and motility and turn into less motile coccoid-like forms.	

9.4.7 Further verification

If further verification is necessary isolates may be speciated (see 9.5) or, alternatively, isolates could be sent to a reference laboratory for species identification and typing by phenotypic or molecular methods.

Be sure to adhere strictly to the instructions for transport given by this laboratory in order to avoid the campylobacters dying due to adverse conditions during transport.

9.5 Identification of *Campylobacter* species (optional)

9.5.1 General

Among the *Campylobacter* spp. growing at 41,5 °C, the most frequently encountered species are *Campylobacter jejuni* and *Campylobacter coli*. *Campylobacter lari* and *Campylobacter upsaliensis* have also been described. The characteristics given in Table 2 permit their differentiation. The isolation of other *Campylobacter* species is unlikely under the culturing conditions described in this document.

9.5.2 Detection of catalase

From each subculture of selected colonies in 9.4.2, deposit a loop of culture into a drop of hydrogen peroxide (see C.7.1) on a clean microscope slide.

The test is positive if bubbles appear within 30 s.

Positive and negative controls should be analysed alongside to confirm the results. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Enterococcus faecalis* WDCM 00085 or WDCM 00087 (negative control).

9.5.3 Detection of hippurate hydrolysis

For each colony selected in 9.4.2, prepare a heavy suspension in a tube of appropriate size containing 0,4 ml of a sodium hippurate solution (see C.8.1), taking care not to incorporate any agar.

Shake in order to mix thoroughly and incubate for 2 h in a water bath (6.1) set at 37 °C or 4 h in an incubator (6.1) set at 37 °C.

After incubation, carefully add 0,2 ml of a ninhydrin solution (C.8.2) on top of the sodium hippurate solution. Do not shake.

Interpret after incubation of an additional 10 min at 37 °C. A dark violet colour indicates a positive reaction and a pale violet colour or no change in colour indicates a negative reaction.

Positive and negative controls should be analysed alongside to confirm the results. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Campylobacter coli* WDCM 00004 (negative control).

NOTE Different inoculum levels can cause false positive/negative results.

9.5.4 Detection of indoxyl acetate hydrolysis

Place a loop full (1 µl) of colony material selected in 9.4.2 on an indoxyl acetate disc (C.9) and add a drop of sterile distilled water. A loopful of colony material is required for a clear reaction.

If the indoxyl acetate is hydrolysed, a colour change to dark blue occurs within 5 min to 10 min. No colour change indicates that hydrolysis has not taken place.

Positive and negative controls should be analysed alongside to confirm the results. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Campylobacter lari* WDCM 00204 (negative control).

9.5.5 Interpretation

Campylobacter species growing at 41,5 °C may be identified at a species level in accordance with Table 2.

Table 2 — Characteristics of *Campylobacter* species

Characteristics	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. lari</i>	<i>C. upsaliensis</i>
Catalase (9.5.2)	+	+	+	– or weak +
Hydrolysis of hippurate (9.5.3)	+ ^a	–	–	–
Indoxyl acetate (9.5.4)	+	+	–	+
+ = positive. – = negative. ^a Some hippurate-negative <i>C. jejuni</i> strains have been reported.				

10 Quality assurance

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

Campylobacter jejuni WDCM 00005 or WDCM 00156, *Campylobacter coli* WDCM 00004 or WDCM 00072 and *Campylobacter lari* WDCM 00204 are suitable positive controls for this method. Suitable negative controls include *Escherichia coli* WDCM 00012 or WDCM 00013, or *Staphylococcus aureus* WDCM 00032 or WDCM 00034 (see Table D.1).

11 Expression of results

If the presence of typical campylobacters or defined species of *Campylobacter* genus are confirmed (see 9.4), report the result as thermotolerant *Campylobacter* or a defined species of the genus *Campylobacter* detected in the sample volume examined.

A semi-quantitative estimate of the numbers present in the sample is made from results with different test volumes (see Annex B).

The proportion of positive and negative enrichment broths obtained from an MPN analysis can be used to derive an MPN count for the volume of sample examined. A calculator that fulfils these requirements is available at <https://standards.iso.org/iso/8199>.

For example, in an eleven tube series using 1 × 500 ml, 5 × 100 ml and 5 × 10 ml where the 500 ml is positive, the 5 × 100 ml are positive and only 2 of the 10 ml are positive, the count is calculated as 54 campylobacters per litre.

It is important that sufficient dilutions are used such that some of the enrichment broths are negative to obtain an MPN count.

12 Test report

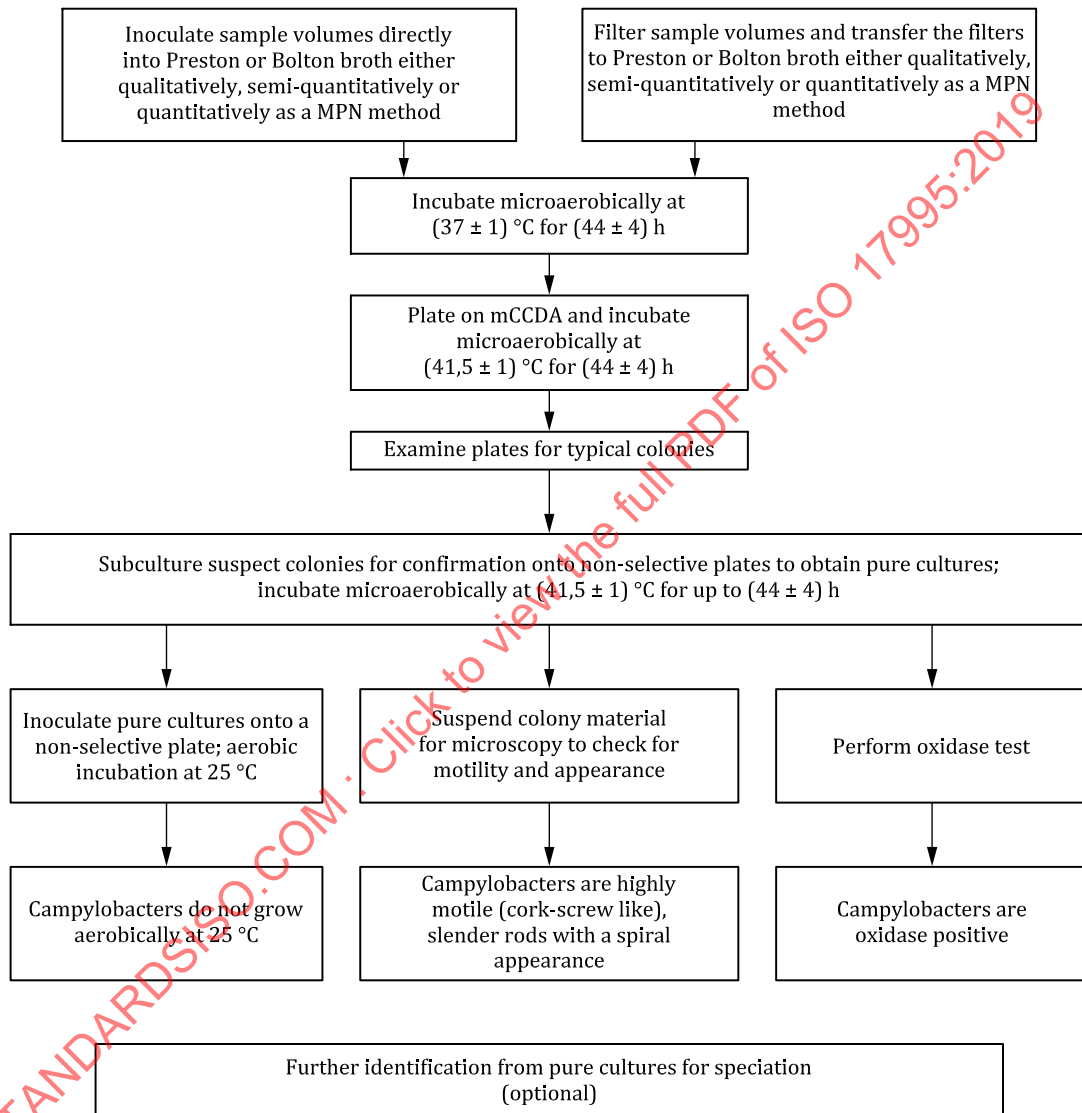
The test report shall include at least the following information:

- a) the test method used, together with a reference to this document, i.e. ISO 17995:2019;
- b) all details necessary for the complete identification of the sample;
- c) the result of the test, as specified in [Clause 11](#);
- d) details of any particular phenomena observed during the analysis and any operation not specified in the method, or considered optional, that could have modified the results.

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Annex A (normative)

Flow diagram of the method



Annex B (normative)

Semi-quantitative analysis

The volume of sample filtered will depend on the number of campylobacters expected in the water. For drinking water, volumes of 10 ml, 100 ml and 1 000 ml are recommended. The smallest volume is filtered first.

For water thus analysed, the result is given as a semi-quantitative estimate per 1 000 ml. *Campylobacter* counts in larger volumes, for example, 5 000 ml can be determined by testing sample volumes of 50 ml, 500 ml and 5 000 ml. Count as positive any test volume which results in the demonstration of campylobacters present in at least one of the two enrichments where both enrichments are used. Estimate the number of campylobacters present in the sample material from [Table B.1](#). An atypical outcome should always result in a scrutiny of all the steps of the analysis to check for possible sources of error.

Table B.1 — Numbers of campylobacters present in sample as a function of the results obtained on the test volumes

Sample volume tested			Semi-quantitative estimate per 1 000 ml
1 000 ml	100 ml	10 ml	
–	–	–	<1 cfu ^a
+	–	–	≥1 cfu but <10 cfu
–	+	–	≥1 cfu but <10 cfu ^b
+	+	–	≥10 cfu but <100 cfu
+	–	+	≥10 cfu but <100 cfu ^b
+	+	+	≥100 cfu
–	+	+	≥100 cfu ^c
–	–	+	≥100 cfu ^c

+ = Test volume *Campylobacter*-positive.
 – = Test volume *Campylobacter*-negative.
^a cfu = colony-forming units
^b Depending on the number of campylobacters in the sample, there is up to 10 % probability that this atypical outcome is caused by mere coincidence.
^c This outcome can be seen when large numbers of competing microorganisms in the sample inhibit the growth of campylobacters.

Annex C (normative)

Composition and preparation of culture media and reagents

C.1 General

The general specifications of ISO 11133 are applicable to the preparation of the culture media described in this annex. If culture media or reagents are prepared from dehydrated media and supplements/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been determined in some studies and are only indicative. The user should verify these under their own storage conditions (as specified in ISO 11133).

Performance testing of culture media is described in [Annex D](#).

C.2 Complete Preston broth (Reference [3])

Special meat extract ^a		10,0 g
Peptone		10,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Polymyxin B sulfate	(CAS No. 1405-20-5)	5 000 IU
Rifampacin	(CAS No. 13292-46-1)	0,01 g
Trimethoprim lactate salt	(CAS No. 23256-42-0)	0,01 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,25 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Lysed blood		50 ml
Water		950 ml

^a Lab-Lemco powder (OXOID) is a trade name of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

NOTE Double strength medium is prepared by adding twice the amount of materials to 900 ml of water. The medium is completed by the addition of 100 ml of lysed defibrinated or citrate-stabilized blood from cattle, sheep or horses.

C.2.1 Preston basal broth

C.2.1.1 Composition

Special meat extract ^a		10,0 g
Peptone		10,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Water		940 ml

^a Lab-Lemco powder (OXOID) is a trade name of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

C.2.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, heating if necessary. Adjust the pH, if necessary, so that, after sterilization, the pH of the complete medium is $7,4 \pm 0,2$ at 25 °C.

Dispense 470 ml aliquots in bottles of suitable capacity with screw caps. Make allowance for losses during sterilization. Sterilize in an autoclave at (121 ± 3) °C for (15 ± 3) min. Open the autoclave as soon as it has cooled to a safe temperature (for example 70 °C) and tighten the screw caps to avoid inclusion of air.

In airtight bottles, the basal broth shall be stored at (5 ± 3) °C for not more than six months (see [6.11](#)).

C.2.2 Solution of Preston *Campylobacter*-selective supplement

C.2.2.1 Composition

Polymyxin B sulfate	(CAS No. 1405-20-5)	5 000 IU
Rifampicin	(CAS No.13292-46-1)	0,01 g
Trimethoprim lactate salt	(CAS No. 23256-42-0)	0,01 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Ethanol, 95 % (volume fraction)		5 ml

C.2.2.2 Preparation

Dissolve the components in the ethanol.

C.2.3 Solution of *Campylobacter* growth supplement (References [\[4\]](#), [\[6\]](#))

C.2.3.1 Composition

Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,25 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Water		5 ml

C.2.3.2 Preparation

Dissolve the components in the water and sterilize by filtration. Aliquots of 5 ml should be stored at $(-20 \pm 5) ^\circ\text{C}$ for not more than twelve months.

C.2.4 Lysed blood

Use defibrinated or citrate-stabilized blood from cattle, sheep or horses. The blood should be stored for not more than one month at $(5 \pm 3) ^\circ\text{C}$, provided it shows no signs of deterioration.

The blood is lysed by freezing at $(-20 \pm 5) ^\circ\text{C}$ overnight. Portions of lysed blood should be stored in tight vials at $(5 \pm 3) ^\circ\text{C}$ for not more than 3 d. Frozen blood should be stored for not more than six months, (see Reference [6]).

C.2.5 Preparation of complete Preston broth

C.2.5.1 Composition

Basal broth (C.2.1)	940 ml
Preston <i>Campylobacter</i> -selective supplement (C.2.2)	5 ml
Preston <i>Campylobacter</i> growth supplement (C.2.3)	5 ml
Lysed blood (C.2.4)	50 ml

C.2.5.2 Preparation

Broth and supplements are mixed immediately prior to carrying out inoculations. The *Campylobacter* growth supplement (see C.2.3) and the lysed blood (see C.2.4) are the last ingredients to be added. After tightening the screw caps to minimize uptake of oxygen, the complete broths are gently rotated.

The complete medium should be stored at $(5 \pm 3) ^\circ\text{C}$ for not more than 7 d (see Reference [6]).

C.3 Complete Bolton broth (Reference [2])

Meat peptone		10,0 g
Lactalbumen hydrolysate		5,0 g
Yeast extract		5,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
α -Ketoglutaric acid monopotassium salt	(CAS No. 328-50-7)	1,0 g
Sodium pyruvate	(CAS No. 113-24-6)	0,5 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,5 g
Sodium carbonate anhydrous	(CAS No. 497-19-8)	0,6 g
Haemin (dissolved in 0,1 % sodium hydroxide)	(CAS No. 16009-13-5)	0,01 g
Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,02 g
Vancomycin hydrochloride	(CAS No. 1404-93-9)	0,02 g
Trimethoprim lactate salt	(CAS No. 23256-42-0)	0,02 g

Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Lysed blood		50 ml
Water		950 ml

NOTE Double strength medium is prepared by adding twice the amount of materials to 900 ml of water. The medium is completed by the addition of 100 ml of lysed defibrinated or citrate-stabilized blood from cattle, sheep or horses.

C.3.1 Bolton basal broth

C.3.1.1 Composition

Meat peptone		10,0 g
Lactalbumin hydrolysate		5,0 g
Yeast extract		5,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
α -Ketoglutaric acid, monosodium salt	(CAS No. 328-50-7)	1,0 g
Sodium pyruvate	(CAS No. 113-24-6)	0,5 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,5 g
Sodium carbonate anhydrous	(CAS No. 497-19-8)	0,6 g
Haemin (dissolved in 0,1 % sodium hydroxide)	(CAS No. 16009-13-5)	0,01 g
Water		945 ml

C.3.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, heating if necessary. Adjust the pH, if necessary, so that, after sterilization, the pH of the complete medium is $7,4 \pm 0,2$ at 25 °C.

Dispense 475 ml aliquots in bottles of suitable capacity with screw caps. Make allowance for losses during sterilization. Sterilize in an autoclave at (121 ± 3) °C for (15 ± 3) min. Open the autoclave as soon as it has cooled to a safe temperature (for example 70 °C) and tighten the screw caps to avoid inclusion of air.

In airtight bottles, the basal broth should be stored at (5 ± 3) °C for not more than six months in the dark (see [6.11](#)).

C.3.2 Solution of Bolton broth selective supplement

C.3.2.1 Composition

Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,02 g
Vancomycin hydrochloride	(CAS No. 1404-93-9)	0,02 g
Trimethoprim lactate	(CAS No. 23256-42-0)	0,02 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Ethanol/sterile distilled water 50/50 (volume fraction)		5 ml

C.3.2.2 Preparation

Dissolve the components in the 50/50 mixture of ethanol and sterile distilled water.

C.3.3 Preparation of complete Bolton broth

C.3.3.1 Composition

Bolton basal broth (C.3.1)	945 ml
Bolton broth selective supplement (C.3.2)	5 ml
Lysed blood (C.2.4)	50 ml

C.3.3.2 Preparation

Broth and supplements are mixed immediately prior to carrying out inoculations. The lysed blood (see [C.2.4](#)) is the last ingredient to be added. After tightening the screw caps to minimize uptake of oxygen, the complete broths are gently mixed.

The complete medium should be stored at $(5 \pm 3) ^\circ\text{C}$ (see [6.11](#)) for not more than 7 d (see Reference [\[6\]](#)).

C.4 Complete modified-charcoal cefoperazone desoxycholate agar (mCCDA) (Reference [\[5\]](#))

Special meat extract ^a		10,0 g
Peptone		10,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Activated charcoal	(CAS No. 7440-44-0)	4,0 g
Casein hydrolysate		3,0 g
Sodium desoxycholate	(CAS No. 302-95-4)	1,0 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,032 g

Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Agar		12,0 g
Water		1 000 ml

^a Lab-Lemco powder (OXOID) is a trade name of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

C.4.1 Basal medium

C.4.1.1 Composition

Special meat extract ^a		10,0 g
Peptone		10,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Activated charcoal	(CAS No. 7440-44-0)	4,0 g
Casein hydrolysate		3,0 g
Sodium desoxycholate	(CAS No. 302-95-4)	1,0 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Agar		12,0 g
Water		1 000 ml

^a Lab-Lemco powder (OXOID) is a trade name of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

C.4.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, by bringing to the boil. Adjust the pH, if necessary, so that, after sterilization, the pH of the complete medium is $7,4 \pm 0,2$ at 25 °C.

Dispense the basic medium into bottles of suitable capacity with screw caps. Make allowance for losses during sterilization. Sterilize in an autoclave at (121 ± 3) °C for (15 ± 3) min. Open the autoclave as soon as it has cooled to a safe temperature (for example 70 °C) and tighten the screw caps to avoid inclusion of air.

In airtight bottles, the basal medium should be stored at (5 ± 3) °C (see 6.11) for not more than one month in the dark.

C.4.2 mCCDA selective supplement

C.4.2.1 Composition

Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,032 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Water		5 ml

C.4.2.2 Preparation

Dissolve the components in the water. Sterilize by filtration.

C.4.3 Preparation of complete mCCDA

C.4.3.1 Composition

Basal medium (C.4.1)	1 000 ml
Antibiotic solution (C.4.2)	5 ml

C.4.3.2 Preparation

Cool molten basal medium (see [C.4.1](#)) to $(48 \pm 3) ^\circ\text{C}$. Aseptically add the mCCDA selective supplement ([C.4.2](#)). Mix carefully and pour into sterile Petri dishes ([6.9](#)). Allow to solidify. Immediately before use, dry the agar plates.

Plates of mCCDA in airtight bags should be stored at $(5 \pm 3) ^\circ\text{C}$ in the dark for not more than 10 d (see [6.11](#) and Reference [\[6\]](#)).

NOTE Selective principles in mCCDA in this case mainly refer to the use of third generation β -lactams, for example, cefoperazone. Examples of alternative media are Preston agar^[3], Butzler agar^[7], etc. More information on media can be found in Reference [\[6\]](#).

C.5 Columbia agar (with and without blood)

C.5.1 Basal medium

C.5.1.1 Composition

Enzymatic digest of animal tissue		23,0 g
Starch soluble	(CAS No. 9005-84-9)	1,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Agar		12,0 g
Water		1 000 ml

C.5.1.2 Preparation

Dissolve the basic components or the dehydrated complete medium in the water, by bringing to the boil. Adjust the pH, if necessary, so that after sterilization, the pH of the complete medium is $7,4 \pm 0,2$ at $25 ^\circ\text{C}$.