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Water quality — Enumeration of *Legionella*

Qualité de l'eau — Dénombrement des Legionella

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Contents

Page

Foreword

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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).

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

This second edition of ISO 11731 cancels and replaces ISO 11731:1998 and ISO 11731-2:2004, which have been technically revised.

Introduction

After the first recognized outbreak of Legionnaires' disease in 1976, the isolated bacterium was named *Legionella pneumophila*. Legionellae are widely found in natural and artificial aquatic environments, soils, composts and can cause legionellosis. Legionellae can grow intracellularly in protozoa like *Acanthamoeba castellanii*, *Hartmannella* species or *Naegleria* species. At least 61 different *Legionella* species have been described. In 26 of these species, some strains infecting humans have been reported. *Legionella pneumophila* can be subtyped into at least 15 different serogroups; nine other species also can be subtyped into at least two separate serogroups. Monitoring for legionellae is important for public health reasons to identify environmental sources which can pose a risk of legionellosis, such as evaporative cooling towers, hot- and cold-water distribution systems in buildings and associated equipment such as spa pools, dental units, air conditioning units, etc. Monitoring is also important for validation of control measures and ongoing verification that controls remain effective.

Water quality — Enumeration of *Legionella*

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 of this document 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 and competent staff.

1 Scope

This document specifies culture methods for the isolation of *Legionella* and estimation of their numbers in water samples.

These methods are applicable to all kinds of water samples including potable, industrial, waste and natural waters. These methods can be used for water related matrices, e.g. biofilms, sediments, etc.

Not all *Legionella* species are culturable; therefore, the methods described in this document do not recover all species of *Legionella*.

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 3696, *Water for analytical laboratory use — Specification and test methods*

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

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms 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:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Legionella
genus of microorganisms normally capable of growth on buffered charcoal yeast extract (BCYE) agar containing L-cysteine and iron(III) salts

Note 1 to entry: For a more detailed description of *Legionella* species, see Annex A.

4 Principle

4.1 General

Legionellae in the water sample are concentrated by membrane filtration, diluted or directly plated depending on the origin/characteristics of the sample. The desired level of detection can vary depending on (inter)national legislation and the reason for sampling or investigation. Samples expected to contain high numbers of legionellae, such as those obtained during outbreak investigations, can be processed with and/or without the concentration steps. To reduce the growth of the concentrated non-target bacteria, which can interfere with the recovery of the target legionellae, portions of the water samples are also subjected to heat treatment, acid treatment or a combination of both treatments.

Dilution is necessary when high concentrations of *Legionella* and/or other bacteria are expected. Separate portions of the diluted sample should be pre-treated; one with heat and a second with acid solution or, in case of extremely contaminated samples, with a combination of acid solution and heat before culturing on selective media.

Treated and/or untreated portions of the sample are transferred onto plates of the chosen culture medium selective for *Legionella* and incubated.

NOTE Mechanical treatment of the sample can enhance the recovery of *Legionella*.

4.2 Examination

After incubation, morphologically characteristic colonies on the selective culture media are regarded as presumptive *Legionella*.

4.3 Confirmation

Presumptive colonies are confirmed as *Legionella* by subculture to demonstrate their growth requirement for L-cysteine and iron(III).

NOTE If species and serotype identification are requested, further tests are needed (see Annex G). These tests are not part of the standardized methods described in this document.

5 Apparatus and glassware

Usual laboratory equipment and in particular:

5.1 Sterile Petri dishes.

5.2 Incubator, capable of being maintained at (36 ± 2) °C.

5.3 Ultraviolet lamp, emitting light of wavelength (360 ± 20) nm.

5.4 Membrane filtration equipment, suitable for filtering water volumes of 10 ml up to 1 000 ml.

5.5 Membrane filter.

5.5.1 Membrane filter for concentration and elution, polycarbonate or polyethersulfone membrane filters, diameter 47 mm to 142 mm with rated pore sizes of 0,2 µm; see Reference [6]. These types of membrane filters are used for concentration followed by a washing procedure.

5.5.2 Membrane filter for direct placing on culture media, membrane filters from cellulose nitrate or mixed cellulose esters, diameter 47 mm to 50 mm with rated pore sizes of 0,2 µm or 0,45 µm. These

types of membrane filters are used for direct placing onto the culture media after filtration. Filters shall be evaluated prior to use in accordance with ISO 7704.

NOTE Black membrane filters contrast better with the white *Legionella* colonies than light-coloured membrane filters.

5.6 pH meter, with an accuracy of ± 0,1 at 20 °C to 25 °C.

5.7 Vortex mixer.

5.8 Ultrasonic water bath, suitable for ensuring that the level of diluent covering the membrane filter is below the level of water in the water bath.

5.9 Water bath, capable of being maintained at (50 ± 1) °C.

5.10 Glassware, sterilized according to ISO 8199.

5.11 Dissection microscope, stereoscopic, with magnification of at least 4× and with oblique incident illumination.

NOTE Also, a hand lens (magnification at least 4×) can be used.

5.12 Disinfected forceps, for handling of membrane filters.

NOTE Forceps with round ends are generally used in order not to damage the membrane during handling.

5.13 Screw cap sterile container, with or without sterile glass beads. To ensure maximum removal of the legionellae from the membrane filter, sterile glass beads (diameter 2 mm to 3 mm) can be added to the sterile container. Add sufficient glass beads to the sterile container just enough to cover the bottom of the container.

6 Culture media and reagents

Use chemicals of analytical grade in the preparation of culture media and reagents unless otherwise stated (see the Note). Prepare the culture media and reagents according to the instructions given in Annexes B, C and D. Prepare culture media using distilled or demineralized water, which is free from substances that might affect growth of microorganisms under the test conditions. The water shall comply with the requirements of ISO 3696, grade 3.

Alternatively, use commercially available culture media and reagents prepared and used according to the manufacturer's instructions.

NOTE Chemicals of other grades can be used, providing they are shown to be of equal performance in the test.

6.1 Culture media.

See Annex B.

6.1.1 Buffered charcoal yeast extract (BCYE) agar.

See B.1.

6.1.2 Buffered charcoal yeast extract agar without L-cysteine (BCYE–cys).

See B.2.

NOTE Blood agar (see B.6), nutrient agar (see B.7) or tryptone soy agar (see B.8) can be used instead of BCYE–cys agar.

6.1.3 Buffered charcoal yeast extract agar with selective supplements (BCYE+AB).

See B.3.

6.1.4 Glycine vancomycin polymyxin B cycloheximide (GVPC) agar.

See B.4.

6.1.5 Modified Wadowsky Yee (MWY) agar.

See B.5.

6.2 Diluents.

See Annex C.

6.2.1 Page's saline.

See C.1.

6.2.2 Diluted Ringer's solution.

See C.2.

6.3 Acid solution.

See Annex D.

7 Sampling

Carry out sampling, transport and storage of the samples in accordance with ISO 19458. Take care not to expose the samples to adverse temperature conditions (e.g. freezing or overheating).

NOTE The use of insulated containers is helpful in this regard.

8 Procedure

8.1 Samples

Due to the complex nature of different sample matrices, the laboratory shall determine the appropriate method for each sample type. The decision matrix is provided in Annex J to determine which appropriate method shall be undertaken. Annex J describes the requirements and provides additional options.

In order to ensure the detection of legionellae from water samples, a concentration technique by membrane filtration (see 8.2.2 or 8.2.3) will be required in most cases. Where the concentration of legionellae is expected to be greater than 10^4 colony forming units per litre (cfu/l), direct plating of the unconcentrated sample can also be carried out. For highly contaminated samples, dilute (refer to Annex C for suitable diluents) and use direct plating before and after the pre-treatment (see 8.3). Record volumes of sample diluted or processed and which pre-treatment(s) has (have) been applied.

When the number of legionellae in any given sample is not known, concentration techniques are usually performed. Therefore, follow the procedure described in 8.2.2 or 8.2.3.

8.2 Concentration of water samples

8.2.1 General

For a general description of the membrane filtration technique, see ISO 8199. Filtration can be done by vacuum filtration or positive pressure filtration.

The flow rate should be adjusted so as not to exceed the maximum specified by the manufacturer for the filter size or type.

NOTE The procedure for water related matrices (swabs, sediment, etc.) is described in Annex I.

8.2.2 Membrane filtration and direct placing of the membrane filter on culture media

Filter the water sample (without treatment, after acid treatment and, if required, after heat treatment) through a cellulose nitrate or mixed cellulose esters membrane filter (5.5.2). The acid treatment can also be done directly on the membrane filter in the funnel (see 8.3.2). The volume filtered depends on the particulate content of the water or the desired detection level. The filtered volume of the sample shall be recorded. Carefully remove the membrane filter from the stand with disinfected forceps (5.12) and place it (right-side up) directly on the culture media, ensuring that no air bubble is trapped underneath.

NOTE Where concentration by filtration is not possible (e.g. due to a high level of deposit), the sample can be concentrated by centrifugation (see Annex F).

8.2.3 Membrane filtration followed by a washing procedure

Filter the water sample through a polycarbonate or polyethersulfone membrane filter (5.5.1). The volume filtered depends on the particulate content of the water or the desired detection level. The filtered volume of the sample shall be recorded. Remove the membrane filter from the stand with disinfected forceps (5.12). Work carefully to avoid loss of residual deposit. Place the membrane filter (right-side down) in a screw cap sterile container with or without sterile glass beads (5.13). To wash the microorganisms from the membrane filter, add 5 ml to 10 ml of sterile diluent (see Annex C) or sample, and shake vigorously using a vortex mixer (5.7) for at least 2 min. Alternatively, place the container (5.13) in an ultrasonic water bath (5.8) for a time interval that has been verified to determine the optimum time interval for maximum recovery. Ensure that the level of diluent covering the membrane is below the level of water in the ultrasonic water bath.

This concentrate represents the prepared sample. Record the volume of the concentrate. Membrane filters may be cut into pieces using sterile scissors to aid elution.

Divide the concentrate into three portions. Use one portion untreated, one portion for treatment with heat (see 8.3.1) and one portion for treatment with acid solution (see 8.3.2).

NOTE 1 Alternatively, the scraping or rubbing technique can be used for removal of the bacteria from the membrane filter (see Annex E).

NOTE 2 Where concentration by filtration is not possible (e.g. due to a high level of deposit), the sample can be concentrated by centrifugation (see Annex F).

NOTE 3 An additional membrane filtration can be used for the acid pre-treatment directly on the membrane filter in the funnel.

8.3 Sample pre-treatment

8.3.1 Heat treatment

Add the sample (concentrated or unconcentrated) to a sterile container and place it in a water bath (5.9) at (50 ± 1) °C for (30 ± 2) min. Small volumes (≤ 5 ml) should be used to ensure a short period until the desired temperature is reached. If many samples are treated together or large sample volumes are treated or thick-walled containers are used, monitor the temperature in a separate container similar to that used for the sample. The time starts when the required temperature is reached. Large sample volumes or thick-walled containers should be cooled to avoid overheating after being removed from the water bath.

8.3.2 Acid treatment

Dilute one volume of the sample (concentrated or unconcentrated) with nine volumes of the acid solution (see Annex D), mix well and leave it for (5,0 ± 0,5) min. If the diluted acid treated sample is used for the calculation of the final concentration of *Legionella* species in the sample, the dilution should be factored. Volumes greater than 0,1 ml can be plated to decrease the limit of detection.

Acid treatment can also be done directly on the membrane filter in the funnel. Transfer around 30 ml acid solution (see Annex D) onto the membrane filter. Leave it for (5 ± 0,5) min and remove the acid solution by filtration. Wash the membrane filter with at least 20 ml of the diluent (see Annex C). It is important that the diluent does not rinse the surface of the funnel that had not been in contact with the acid solution.

8.4 Culture

8.4.1 General

The choice of the method used for the enumeration of *Legionella* species depends on the origin/characteristics of the sample and the reason of sampling or investigation. An assumption shall be made about the expected concentration of interfering microorganisms based on experience or origin of the sample. Also, the desired lower limit of detection level needs to be considered. A decision matrix for choosing an appropriate method is described in detail in Annex J.

Depending on the desired detection level, it is possible to use more than one plate of the different culture media mentioned in the following subclauses.

8.4.2 Samples with a high concentration of *Legionella* species and a low concentration of interfering microorganisms

Plate the sample directly if the number of *Legionella* is expected to exceed 10^4 cfu/l. Inoculate and spread 0,1 ml to 0,5 ml of the sample on one plate of BCYE agar (see B.1) and one plate of BCYE+AB agar (see B.3). Record the inoculated volume.

8.4.3 Samples with a low concentration of *Legionella* species and a low concentration of interfering microorganisms

8.4.3.1 Direct placing of membrane filter on culture media after membrane filtration

Filter the sample (see 8.2.2) and place the untreated membrane filter directly on one plate of BCYE agar (see B.1). The membrane filters treated with acid solution according to 8.3.2 are placed on one or more of the selective or highly selective plates of BCYE+AB agar (see B.3) or GVPC agar (see B.4) or MWY agar (see B.5).

8.4.3.2 Plating after membrane filtration with washing procedure

Inoculate and spread 0,1 ml to 0,5 ml of each concentrated portion of the sample (untreated, heat treated and acid treated) from the membrane filtration with washing procedure (see 8.2.3) on one plate of BCYE agar (see B.1) and on one or more of the selective or highly selective plates of BCYE+AB agar (see B.3) or GVPC agar (see B.4) or MWY agar (see B.5). Record the inoculated volume.

8.4.4 Samples with a high concentration of interfering microorganisms

Culture samples with a high concentration of interfering microorganisms unconcentrated (direct), concentrated (see 8.2) or diluted (1:10). Divide each subsample into three portions. Use one portion untreated, the second portion for treatment with heat (see 8.3.1) and the third portion for treatment with acid solution (see 8.3.2). Inoculate and spread 0,1 ml to 0,5 ml of each portion of the subsamples on one plate of GVPC agar (see B.4) or one plate of MWY agar (see B.5). Record the inoculated volume.

8.4.5 Samples with an extremely high concentration of interfering microorganisms

Culture samples with an extremely high concentration of interfering microorganisms unconcentrated and diluted (1:10 and 1:100) after a pre-treatment with a combination of heat and acid. For the combined treatment first, the heat treatment (see 8.3.1) is done followed by the acid treatment (see 8.3.2). It is important to cool the heat-treated sample to room temperature before the acid treatment is done. Prepare dilutions directly after the acid treatment in sterile diluent (see Annex C).

Mix well by shaking, using a vortex mixer (5.7) or an ultrasonic water bath (5.8). If necessary, add a layer (just enough to cover the bottom of the container) of sterile glass beads to the sample to aid disaggregation of the material. Inoculate and spread 0,1 ml to 0,5 ml of each portion on one plate of GVPC agar (see B.4) or one plate of MWY agar (see B.5). Record the inoculated volume.

8.4.6 Incubation

Allow the inoculated plates to stand until the inoculated volume has been absorbed, then invert the plates and incubate at (36 ± 2) °C for 7 d to 10 d. Create a humid atmosphere to prevent desiccation of the plates, for example, by placing in an enclosed container.

NOTE Validation data using artificially spiked samples have demonstrated no difference in counts between incubation for 7 d and 10 d. Natural samples containing wild strains of *Legionella* can, however, require the full incubation period of 10 d to present growth.

8.4.7 Examination of the plates

Inspect the plates for the first time either on day 2, 3, 4 or 5 followed by a final inspection at the end of the incubation period. This is to identify samples where overgrowth has occurred. The final quantitative result is not available until the end of the incubation period. For the range of quantitative determination, see Table H.1. As *Legionella* grows slowly and can be masked by the growth of other microorganisms, it is also recommended to use the dissection microscope with oblique incident illumination (5.11). Record the number of each type of presumptive *Legionella* colony present.

In case of outbreak investigations, it is advisable for samples expected to have a high concentration of interfering microorganisms to check the plates on day 2 to determine if dilutions are needed. Be aware of the potentially negative impact of the conservation of the concentrate or sample for a period of two additional days.

Colonies of *Legionella* are white-grey in general but can also appear in other colours. They are smooth with an entire edge and exhibit a characteristic ground-glass appearance. Under an ultraviolet lamp (5.3), colonies of several species (*L. anisa*, *L. bozemanii*, *L. cherrii*, *L. dumoffii*, *L. gormanii*, *L. gratiana*, *L. parisiensis*, *L. steigerwaltii* and *L. tucsonensis*) autofluoresce brilliant white; *L. erythra* and *L. rubrilucens* appear red. Colonies of *L. pneumophila* appear dull green often tinged with yellow. The colour of fluorescence can help to differentiate colonies in samples containing different species of *Legionella*. To avoid the possibility that *Legionella* cells could be killed or damaged so that they are not recoverable,