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PROTOCOLS

MICROBIOLOGICAL ANALYSIS IN DRINKING WATER

ALL PROCEDURES ACCORDING TO ISO REGULATIONS

Inspired by knowledge

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What is the importance of Microbiological analysis in drinking water?

From a health risk management point of view, water can be divided into two large groups:

DRINKING WATER: Water that can be publicly distributed (tap water), from subterranean water sources such as springs, wells and rivers, and bottled water.

NON-DRINKING WATER: Water for industrial ice production, washing, and aquaculture, as well as recycled water.

It is therefore essential that water for human consumption is safe. In order to determine that, both its physical-chemical parameters (pH, dissolved oxygen, redox potential, etc.) and microbiological parameters must be analysed.

A **microbiological analysis** does not consist on determining the species of all microorganisms present in the sample, but on searching for pathogens or those microorganisms that accompany them. These marker microorganisms are present in large numbers in the intestine of mammals, making them markers of faecal contamination. Their presence in water would therefore indicate that it is not suitable for human consumption.

Hus, as specified in **RD 140/2003**, for water to be considered safe, it must meet the following microbiological parameters:

1. Not to exceed 100 CFU/ml of **culturable microorganisms**
2. Absence of **total Coliforms** in 100 ml of sample
3. Absence of ***Escherichia coli*** in 100 ml of sample
4. Absence of **Intestinal Enterococci** in 100 ml of sample
5. Absence of ***Clostridium perfringens*** in 100 ml of sample

At **Condalab**, we want to help the different quality control laboratories by making available to them the full range of culture media under **ISO formulations** as well as their different **analysis procedures**.

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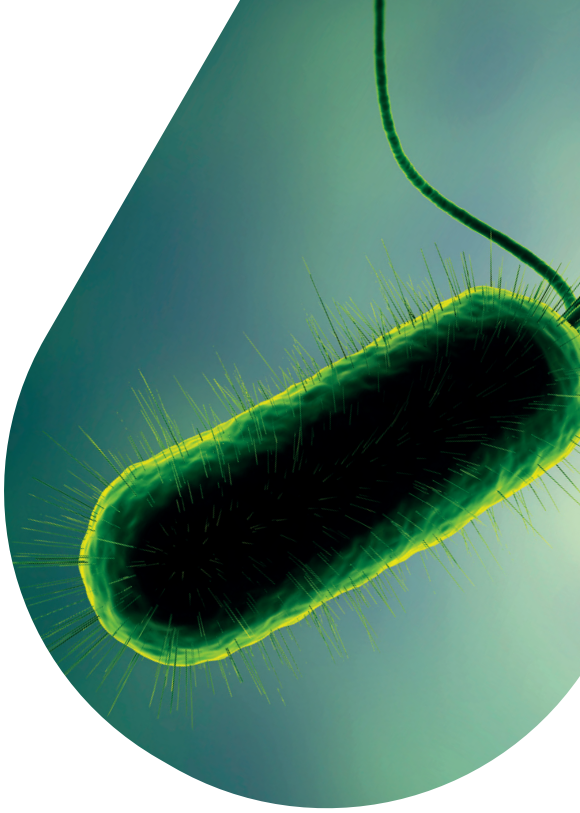
Legionella count

Procedure as defined by ISO 11731:2017



Introduction

Legionella is a bacterium generally shaped as a bacillus ranging from 0.3 to 0.9 µm wide, and from 1.5 to 5 µm in length. They show weak Gram staining (gram-negative) and are motile by one or more polar or subpolar flagella. It is a strict aerobic microorganism; it needs oxygen for its survival and, generally, it is not very active.

These environmental bacteria find their natural niche in surface waters such as lakes, rivers, and ponds, where they are part of the bacterial flora. From these natural reservoirs, these bacteria can colonise supply systems of entire cities, and access the sanitary water systems through the distribution network.

It is currently addressed by *RD 865/2003*, which regulates the health and hygiene criteria for the prevention and control of legionellosis. In practice, prevention is achieved through piping system routine maintenance, monitoring of microbial status, and proper disinfecting processes. The Legionella analysis is the only means to assess risks, so as to determine whether preventive and corrective measures have been sufficient.

Bibliography

BOPP C.A et al. *Isolation of Legionella spp. from environmental water samples by low-pH treatment and use of selective medium*. J. Clin. Microbiol. April 1981, 13 (4) pp. 714-719

DENNIS P.J et al. *Comparison of isolation methods for Legionella spp, Legionella. Proceedings of the 2nd International Symposium*. America Society for Microbiology, 1984 pp 294-296

UNE-EN-ISO 11731:2017. *Water quality. Enumeration of Legionella.*

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1 - SAMPLE WITH A HIGH CONCENTRATION OF *LEGIONELLA* AND A LOW LOAD OF ACCOMPANYING MICROORGANISMS

SELECTIVE ISOLATION

0.1 - 0.5 ml of the sample is streaked directly onto the Legionella BCYE Agar (**CAT. 1311** + **CAT. 6025**) and BCYE + AB Agar

2 - SAMPLE WITH A LOW CONCENTRATION OF BOTH *LEGIONELLA* AND ACCOMPANYING MICROORGANISMS

2.1 MEMBRANE FILTRATION

UNTREATED SAMPLE

SELECTIVE ISOLATION

The filter is placed on Legionella BCYE Agar (**CAT. 1311** + **CAT. 6025**)

ACID PRE-TREATMENT OF THE SAMPLE

SELECTIVE ISOLATION

The filter is placed on Legionella BCYE Agar (**CAT. 1311** + **CAT. 6025**) or Legionella GVPC Agar (**CAT. 1311** + **CAT. 6022**) or Legionella MWY Agar (**CAT. 1311** + **CAT. 6067**)

2.2 MEMBRANE FILTRATION + ELUTION

PRE-TREATMENT

The sample is divided into 3 portions. One is applied an ACID treatment; another one, a HOT treatment; and the third portion is left UNTREATED

SELECTIVE ISOLATION

0.1 – 0.5 ml of the sample is streaked directly onto the Legionella BCYE Agar (**CAT. 1311** + **CAT. 6025**) and onto another culture medium to choose between BCYE + AB Agar, Legionella GVPC Agar (**CAT. 1311** + **CAT. 6022**) or Legionella MWY Agar (**CAT. 1311** + **CAT. 6067**)

3 - SAMPLE WITH A HIGH CONCENTRATION OF ACCOMPANYING MICROORGANISMS

CONCENTRATION / DILUTION

3 subsamples of the original sample are made: Unconcentrated, concentrated, and diluted

PRE-TREATMENT

The sample is divided into 3 portions. One is applied an ACID treatment; another one, a HOT treatment; and the third portion is left UNTREATED

SELECTIVE ISOLATION

0.1 – 0.5 ml of the sample is streaked directly onto Legionella GVPC Agar (**CAT. 1311** + **CAT. 6022**) or Legionella MWY Agar (**CAT. 1311** + **CAT. 6067**)

4 - SAMPLE WITH AN EXTREMELY HIGH CONCENTRATION OF ACCOMPANYING MICROORGANISMS

PRE-TREATMENT

The sample is first subjected to a HOT treatment followed by an ACID treatment

DILUTION

3 subsamples of the pre-treated sample are made: Unconcentrated, concentrated, and diluted.

SELECTIVE ISOLATION

0.1 – 0.5 ml of the sample is streaked directly onto Legionella GVPC Agar (**CAT. 1311** + **CAT. 6022**) or Legionella MWY Agar (**CAT. 1311** + **CAT. 6067**)

COMMON PROCEDURE REGARDLESS OF SAMPLE TYPE

INCUBATION

Plates are incubated at 36 ± 2°C for 7/10 days.

*Growth is observed on days 2, 3, 4, 5 and at the end of incubation

CONFIRMATION

Subcultures are performed in Legionella BCYE Agar (**CAT. 1311** + **CAT. 6022**) and Legionella BCYE wihout Cys Agar (**CAT. 1311**).

Results: Positive growth in BCYE and negative growth in BCYE-cys

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Escherichia Coli and *Coliforms* enumeration

Procedure as defined by ISO 9308-1:2014

Introduction

In the past, water quality tests and data submissions were based on groups of bacteria called total and faecal coliforms. Coliform bacteria are found in the aquatic environment, in soil, and in vegetation. They are also present in large amounts in the faeces of warm-blooded animals. Because coliforms do not normally cause serious diseases and are easy to grow. Their presence is used as a marker for the presence of other pathogenic organisms of faecal origin.

Nowadays, the tests also contemplate the concentration of *Escherichia coli*. This is a bacteria found within the faecal coliform group and is therefore a marker of faecal contamination. Furthermore, as its survival in non-enteric media is limited, it indicates a recent contamination source.

The water consumed as drinking water is analysed to determine, among other parameters, the concentration of *E. coli*. Similarly, wastewater that has been treated and then recycled for irrigation and/or discharged to surface waters must also meet certain levels for it to be considered safe.

Method

FILTRATION

100 ml of sample (250 ml for bottled water) is filtered)

For sample filtrations and dilutions, filter a minimum volume of 10 ml

PRESUMPTIVE ISOLATION

Place the membrane after filtration in Coliform Chromogenic Agar (CCA) (**CAT. 2080**) / Incubation at 36 °C ± 2 °C for 21 ± 3 h

PRESUMPTIVE COLIFORM COUNT

Presumptive coliform colonies are pink/red in colour

CONFIRMATION

Test the presumptive colonies for oxidase (-) and perform a subculture in Tryptone Soy Agar (**CAT. 1138**) on which to perform the same confirmatory test (-)

E. COLI COUNT ß-GALACTOSIDASE + AND ß-GLUCURONIDASE +

Colonies are dark blue/violet in colour

Bibliography

ISO 7218:2007. *Microbiology of food and anima feeding stuffs. General requirements and guidance for microbiological examinations.*

ISO 9308-1:2014. *Water quality. Enumeration of Escherichia coli and coliform bacteria. Part 1: membrane filtration method for water with low bacterial background flora.*

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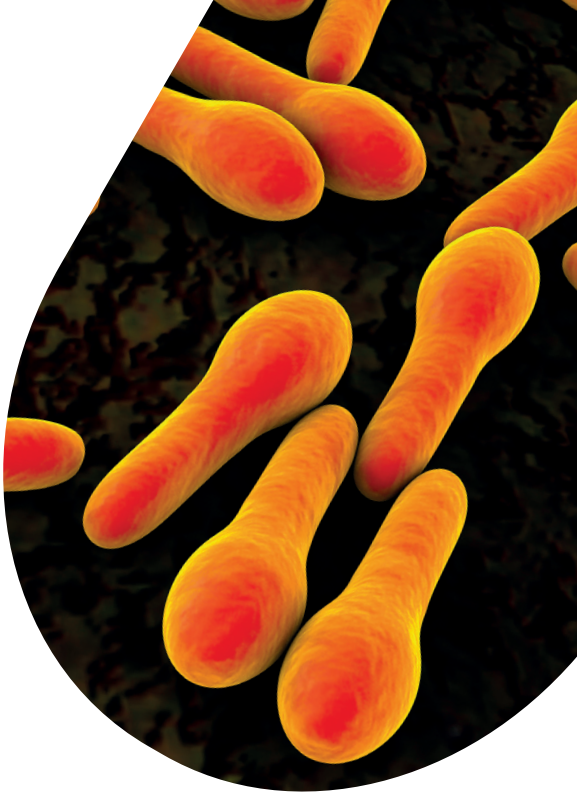
Clostridium Perfringens enumeration by membrane filtration



Procedure as defined by ISO 14189:2013

Introduction

Clostridium perfringens a spore-forming bacteria that is present in human and animal faeces, although they may also originate in other environmental sources. It grows preferably in conditions with very little or no oxygen and, in ideal conditions, it can multiply very rapidly.

Its spores can resist disinfection processes and survive in water much longer than coliforms, which means that this bacterial species can be used to detect older faecal contamination, since as we already know, the *Escherichia coli* is a marker for recent water contamination.

For these reasons, *Clostridium perfringensis* presented as an essential biomarker of water contamination and a very useful marker for water managers on the presence of other pathogens resistant to environmental conditions, such as viruses and protozoan cysts.

For example, in drinking water, its presence is very significant and therefore requires immediate water treatment.

Method

PRE-TREATMENT AND FILTRATION

100 ml of sample is filtered through the membrane

For spore analysis, the sample should be heated to 60 ± 2 °C for 15 min

PRESUMPTIVE ISOLATION

Place the membrane after filtration in T.S.C. Agar (Tryptose Sulfite Cycloserine) (**CAT. 1029** + **CAT. 6020**) Incubation in anaerobiosis at 44 °C ± 1 °C for 21 ± 3 h

ISOLATION OF SUSPECT COLONIES

Suspect colonies are isolated in Columbia Agar (**CAT. 1104**) with blood / Incubation in anaerobiosis at 36 °C ± 2 °C for 21 ± 3 h

CONFIRMATION

Perform the acid phosphatase test on filter paper

Bibliography

BURGER J.S, NUPEN E.M, and GRABOW W.O.K, *Evaluation of four growth media for membrane filtration counting of Clostridium perfringens. Water* S.A. 1984, 10 pp. 521-526

ARAUJO M., SUEIRO R.A, GOMEZ M.J, & GARRIDO M.L. *Enumeration of Clostridium perfringens spores in ground water samples: comparison of six culture media.* J. Microbiol. Methods. 2001, 57 pp. 175-180.

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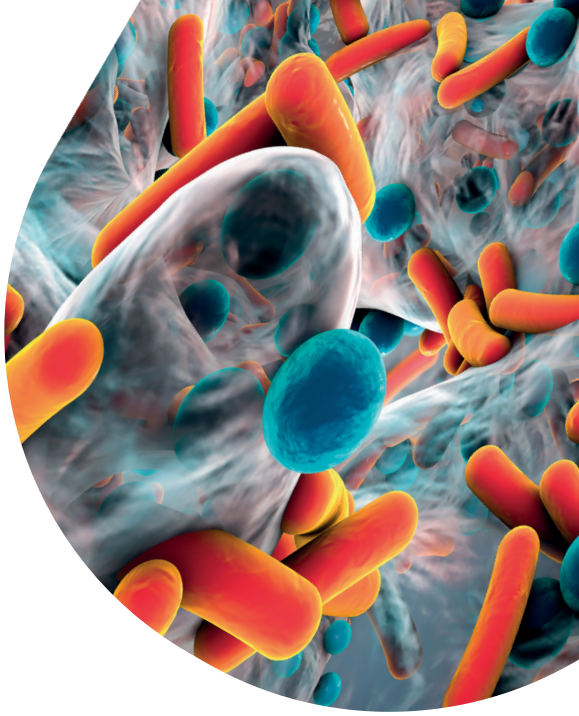
Pseudomonas aeruginosa detection and count



Procedure as defined by ISO 16266:2006

Introduction

Pseudomonas aeruginosa is a ubiquitous species in the environment. Its presence is common in soils and natural water as well as in lakes and rivers. However, they are not often found in drinking water; but when they are present, they are found at low concentrations.

Although the presence of *P. aeruginosa* may be significant in some settings, such as healthcare facilities, there is no evidence that regular applications of drinking water are a source of infection for the general population. This bacterium is sensitive to disinfection, so proper disinfection can minimize its entry into distribution systems.

Therefore, control measures designed to limit the formation of biofilms, such as treatment to optimise the removal of organic carbon, should reduce the proliferation of these microorganisms.

Method

FILTRATION

The sample is filtered as defined by **ISO 8199**

PRESUMPTIVE ISOLATION

Place the membrane after filtration in Pseudomonas CN Agar (**CAT. 1153**) / Incubation at 36 °C ± 2 °C for 44 ± 4 h

Examine growth after 22 h and at the end of incubation

READING UNDER NATURAL LIGHT

a) Blue/green colonies shall be counted as *Pseudomonas aeruginosa*

b) Red/brown colonies shall be considered as presumptive and confirmatory tests shall be performed

READING UNDER UV LIGHT

Fluorescent colonies not producing piocyanin as presumptive *Pseudomonas aeruginosa*

CONFIRMATION

After subculture in Nutrient Agar (**CAT. 1156**), perform:

- Oxidase reaction (+)
- Inoculate on Acetamide broth (+) (**CAT. 1155**)
- Inoculate on King B Medium (+) (**CAT. 1154**)

CONFIRMATION

After subculture in Nutrient Agar (**CAT. 1156**), perform inoculation on:

- Acetamide broth (+) (**CAT. 1155**)

To check incubation times and temperatures, check the technical sheet of the different references

Bibliography

Council Directive 98/83/EC on the quality of water intended for human consumption. *Official Journy of the European Communities*. L330, 1998, pp. 32-53.

ISO 16266:2006. *Water quality. Detection and enumeration of Pseudomonas aeruginosa. Method by membrane filtration.*

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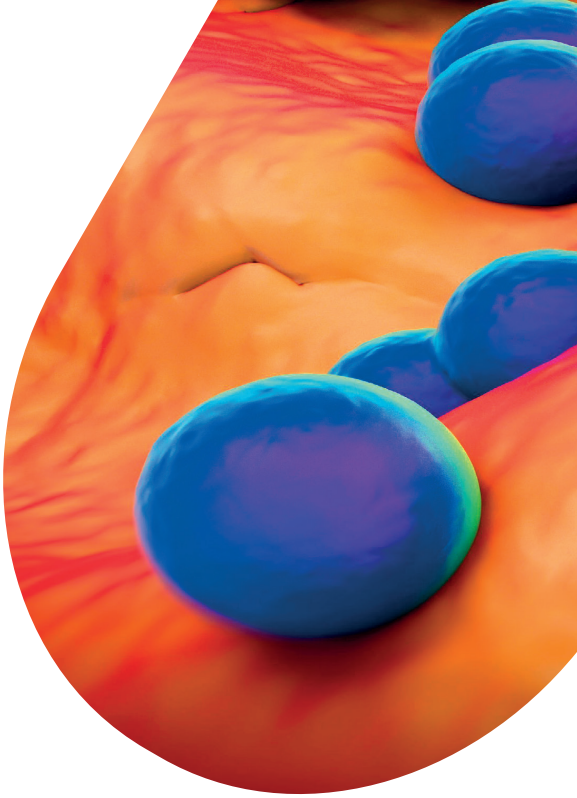
Intestinal Enterococci detection and count



Procedure as defined by ISO 7899-2:2000

Introduction

Enterococci are Gram-positive cocci. They show in the form of pairs or short chains that do not form endospores and are not motile. They are facultative anaerobic microorganisms, chemorganotrophs, with fermentative metabolism. Its optimal growth is at 37 °C. They can grow in the presence of 6,5 % NaCl, at 10 and 45 °C and with a pH 9,6.

Enterococci are part of the normal microbiota of the human gastrointestinal tract and the female genital tract. The most frequent species in clinical isolates are *E. faecalis* and *E. faecium*.

Enterococci may also be present in soil, food, water, plants, animals, and insects, and are often considered good markers of faecal contamination because they are highly resistant to adverse conditions such as freezing and drying.

Some authors consider the genus *Enterococcusas* the most efficient microbial marker to evaluate the quality of seawater for recreational use because it is highly resistant to the saline conditions of this medium. This genus is also directly related to gastroenteritis, respiratory diseases, conjunctivitis, and dermatitis.

Method

FILTRATION

A suitable volume of water is filtered as defined by **ISO 8199**

PRESUMPTIVE ISOLATION

Place the membrane after filtration in Slanetz-Bartley Medium (**CAT. 1109**) / Incubation at 36 °C ± 2 °C for 44 ± 4 h

CONFIRMATION AND ENUMERATION

The suspect colonies are red, brown or pink in colour and convex in shape. Spread these colonies on Bile Esculin Azide Agar (**CAT. 1005**) plates pre-heated to 44 °C / Incubation at 44 ± 0.5 °C for 2 h

The enumeration of colonies shall be done immediately after incubation. Intestinal enterococci are brown in colour with a halo around them

Bibliography

ISO 7704:1985. *Water quality. Evaluation of membrane filters used for microbiological analyses.*

ISO 7899-2:2000. *Water quality. Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.*

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Enumeration of *Culturable Microorganisms*

Procedure as defined by ISO 6222:1999

Introduction

The microbiological markers of water quality are organisms that show a behaviour similar to pathogenic microorganisms whose origin, concentration, habitat and reaction to external factors is that of the majority.

Their presence determines the existence of pathogens and enables the comparison of their reactions to changes in pH and temperature.

They require the identification and quantification of microorganisms by diversity indices adjusted at intervals that qualify water quality and, although the microbiological information obtained from their analysis does not replace physicochemical analyses, it reduces costs and provides information in the monitoring of water quality.

The identification and enumeration of aerobic and mesophilic microorganisms in waters enables:

- Assess the status of water resources at their source
- Determine the efficacy of the treatment process of water intended for human consumption
- Establish the cleanliness level and conditions of the distribution systems
- Detect anomalous changes in the number of microorganisms in the distribution network.

Method

SAMPLE PREPARATION

Conducted according to **ISO 8199** standard

POUR PLATE TECHNIQUE

Add 2 ml of the prepared sample together with 15/20 ml of Yeast Extract Agar (YEA) (**CAT. 1049**)

INCUBATION SERIES 1

Plates are incubated at 36 ± 2 °C for 44 ± 4 h

INCUBATION SERIES 2

Plates are incubated at 22 ± 2 °C for 68 ± 4 h

The count is performed in each of the series of incubated plates for enumeration, expressed as CFU/ml

Bibliography

ISO 6222:1999. Water quality. *Enumeration of culturable microorganism. Colony-count by inoculation in a nutrient agar culture medium.*

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