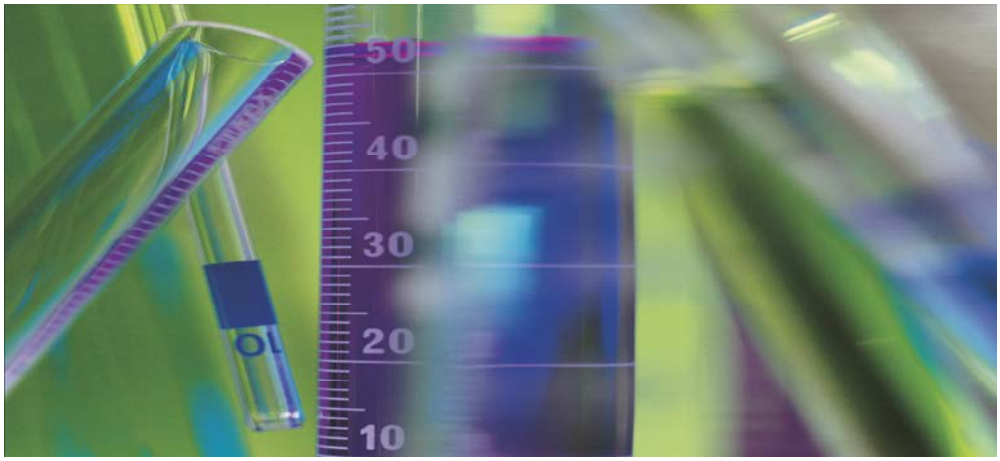
Analytical Method

Detection and identification of bacteria of the genus Legionella

ANALYTICAL METHOD **370**



Applicability
This method is used to detect and ifentifiy bacteria of the genus Legionella in water samples

Standard
No stardard

Sampling system
1 litre (1 L) sterile bottle with thiosulfate

Recommended sampling volume
1 L in a sterile bottle with thiosulfate

Analysis
Growth on BCYE agar media, examination by fibre optic stereomicroscopy, transmitted light microscopy, gas phase chromatography and agglutination test

Minimum reported value (MRV)
Process water: 3000 CFU/L

Higher if dilutions are necessary (meaning presence of interfering or invading heterotrophic bacteria).

Drinking water: 10 CFU/L for filtered 100 mL

Depending on the sample's filterable volume

Range of application
Depending on the dilutions: 1 to 300 colonies per agar

Reliability
Repeatability: 3.5% spread plate method, 6% filtration method

Reproducibility: 5% spread plate method, 7% filtration method

Identification specificity: 100% Legionella by genus and L. pneumophila

Analytical uncertainty (CV_A)
N.A.

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Preamble

The goal of the Act respecting occupational health and safety in Québec is to eliminate at the source, dangers to the health, safety and physical well-being of workers. Permissible exposure values (PEVs) for chemical substances have been established in Schedule I of the Regulation respecting occupational health and safety (ROHS). Section 44 of this regulation, entitled "*Methods,"* specifies that:

> *"…These dusts, gases, fumes, vapours and mists found in the workplace environment shall be sampled and analyzed to obtain an accuracy equivalent to that obtained by applying the methods described in the Sampling Guide for Air Contaminants in the Workplace published by the Institut de recherche Robert-Sauvé en santé et sécurité du travail du Québec..."*

To achieve these objectives, analytical methods for quantifying the workers' degree of exposure are developed and written to establish appropriate means of control. In order to help health and safety professionals in workplaces, the IRSST publishes, periodically revises, and disseminates the *Sampling Guide for Air Contaminants in the Workplace* and the Laboratory Division publishes contaminant analysis methods.

Furthermore, all the terminology used in this method is described in work instruction "I-G-014" of the document management system associated with the IRSST's quality system.

1. PRINCIPLE OF THE METHOD

This method is used to detect and identify bacteria of the genus *Legionella* that can be found in different liquid environments (e.g., water in cooling towers, humidifiers, showers).

It is a growth method obtained by spread plate on BCYE agar (which can contain the BMPA supplement) after dilution or concentration by filtration. The samples may be treated or not with heat to reduce the number of heterotrophic bacteria present in the sample.

2. INTERFERENCES

The sensitivity of this method depends on:

- The capacity to recognize colonies characteristic of *Legionella*.
- The capacity to obtain a pure culture of each type of colony characteristic of *Legionella*, which is necessary for confirmation.
- Compliance with the growth conditions, which promotes the development of *Legionella*.
- The conditions during transport, which can modify the flora of the sample. Heterotrophic bacteria can grow and interfere with the detection of *Legionella* and the latter can lose its capacity for growth. The samples must be stored at between 6° and 20°C.
- The presence of chlorine or biocides in high concentration during water sampling can inhibit the growth of *Legionella* in the laboratory and result in an underestimation.
- The frequency of addition of water treatment products. If it less than 48 hours, the sample must be collected just prior to the next addition of treatment product.
- The presence of heterotrophic bacteria in the sample, which can interfere with the growth of the bacteria of the genus *Legionella.* For example, the presence of *Pseudomonas aeruginosa* bacteria inhibits the growth of *Legionella* on agar. A remark is included in the analytical report when heterotrophic bacteria in large concentration are observed.
- The presence of invading microorganisms developing on the agar, which can make the detection, enumeration and isolation of *Legionella* difficult, and even impossible. A remark must be included in the analytical report when invading bacteria interfere with the analysis.
- Heat treatment, which can reduce the capacity for growth of *Legionella.* A remark about possible underestimation of the concentration of *Legionella* must be included in the analytical report when heat treatment is used.
- The method's selectivity depends on the species of *Legionella that can* be confirmed by *Instant FAME* identification methods from the MIDI Inc.® company and the agglutination tests from the OXOID® company (the appendix contains the lists for each method).

3. MATERIAL

- Inoculation loop
- 20 X-120 X stereomicroscope
- Fibre optic light source
- Gas phase chromatograph with ultra 2 type capillary column (Agilent 19091B-102)
- Sherlock database for *Instant FAME* (MIDI, Delaware, U.S.)
- Refrigerator at constant temperature (5°C ± 3°C)
- CO_2 incubator (35°C ± 1°C, 2.5% CO_2)
- Laminar flow hood
- Chemical hood
- Black nitrocellulose filters (Milipore), porosity of 0.45 µm, dimension of 47 mm
- Vacuum filtration system
- Water bath (50°C ± 2°C)
- Spreaders or automated system for spreading (Easy Spiral dilute)
- Micropipettes
- Sterile tips
- Sterile tubes
- Vortex
- GC vial
- Balance
- Autoclave
- U.V. lamp at 365 nm

4. REAGENTS

- BCYE agars
- BMPA supplement (Cefamandole (4.0 mg), Polymyxin B (80,000 IU), Anisomycin (80 mg) per litre of medium)
- TSA-blood or TSA agar
- Acid buffer (HCL)
- Sterile phosphate buffer (dilution and rinsing water)
- Oxoid® agglutination tests (Legionella pneumophila serogroup 1; 2-14 and Legionella sp)
- Immersion oil
- *Instant FAME* extraction reagents

5. SAMPLING

The samples must be collected in a sterile container. If the collected liquid contains chlorine and/or bromide or any other biocide, thiosulfate must be present in the container (final concentration of 200 mg/L). In principle, 180 mg of sodium thiosulfate pentahydrate will neutralize 1 L of water containing up to 50 mg of chlorine. In the case of sampling performed after a shock treatment, there must be a minimum waiting time of 48 hours between treatment and the collection of the sample. In some cases, to reduce peripheral contamination, the water may have to be run for 2 to 3 minutes before collecting the sample if it is collected

from a valve. For cooling tower water sample, it is important that the sample be representative of the water in the tower and not be makeup water or water from deposits present in the purge valve. The sampler must understand the operation of the tower in order to ensure a representative sample.

It is not necessary to provide a control sample.

For more information on cooling tower sampling, it is useful to consult the document entitled *Protocole d'échantillonnage de l'eau du circuit des tours de refroidissement pour la recherche des légionelles, DR-09-11*, published by the Centre d'expertise en analyse environnementale du Québec (CEAEQ). For other types of water, it may be useful to also consult the document from the Environment Agency of England entitled *The determination of Legionella bacteria in waters and other environmental samples - Part 1 - Rationale of Surveying and Sampling - Methods for the Examination of Waters and Associated Materials*, and the French standard AFNOR FD T 90-522 (2006) entitled *Guide technique de prélèvement pour la recherche de Legionella dans les eaux*.

Transport to the laboratory must be done as soon as possible. It must never exceed 48 hours. It is preferable to allow the samples to cool to room temperature and to protect them from freezing and extreme temperatures. Store them between 6˚C and 20°C during transport. Coolers are necessary only during summer. A note will be entered if the sample cannot arrive within 48 hours. In such a case, the results may no longer be representative of the sample during sampling (underestimation or overestimation).

6. ANALYTICAL PROTOCOL

6.1 Solution preparation

6.1.1 Dilution water

Prepare the 1 X phosphate buffer by performing the necessary dilutions according to the manufacturer's directions.

6.1.2 Acid buffer (HCL)

Prepare a solution of hydrochloric acid (HCl) at 0.2 mol/L (Solution A):

- Add 17.4 mL of concentrated HCl (ρ = 1.18, minimum content 35.4%) or 20 mL of HCl (ρ = 1.16, minimum content 31.5%) to 1 L of distilled water.
- Sterilize by autoclave (121° ± 3°C) for 15 ± 1 min.

Prepare a solution of potassium chloride (KCl) at 0.2 mol/L (Solution B):

- Dissolve 14.9 g of KCl in 1 L of distilled water.
- Sterilize by autoclave (12° ± 3°C) for 15 ± 1 min.
- Mix 3.9 mL of Solution A and 25 mL of Solution B.
- Adjust the pH to 2.2 ± 0.2 by adding a solution of potassium hydroxide (KOH) at 1 mol/L.
- Store in a capped glass container, in the dark, and at ambient temperature for a maximum of one month.

6.1.3 Rinsing solution

Prepare the 1 X phosphate buffer by performing the necessary dilutions according to the manufacturer's recommendations.

6.2 Preparation of analytical controls

For each series of analyses, positive and negative controls must be prepared. The same steps specified for the analyzed samples (heat treatment, acid treatment and filtration if applicable) must be followed for the *Legionella pneumophila* suspension (positive control) and the sterile water solution (negative control). Note: The controls are smeared on a single agar.

6.2.1 Positive control

The positive control is produced by the addition of cells of *Legionella pneumophila* to 1 L of sterile saline water.

Use *Legionella pneumophila* cultured for 3 to 7 days.

- Isolated strains of field sample sometimes show a better recovery for the treatments (heat and acid) than strain ATCC 33155. Such a strain can be used if identification and resistance to treatments are demonstrated.

Prepare 2 mL of a bacterial suspension from a colony of a 3- to 5-day culture of *Legionella pneumophila* on agar.

- The resulting density allows isolated colonies to be obtained on the positive control agars with and without treatment (heat, acid).
- The bacteria of the positive control must be collected in the 3rd or 4th quadrant. This is important to not collect where there is a confluence of colonies because the estimation of the concentration of cultivable cells is more difficult there. The use of isolated colonies results in a better estimation of the final concentration inoculated on agar.

Take 1 mL of this suspension and add it to 1 L of sterile water.
Shake well to homogenize.

The positive control must undergo the same treatment as the samples.

Spread or filtrate 100 µL on the BCYE agars (upon the method used) of the untreated positive control as well as for each treatment. The controls are not spread in triplicate.

6.2.2 Negative control

The negative control consists of the spreading or filtration of sterile water.

6.3 Preparation of samples

6.3.1 Process samples (e.g., cooling tower, pond, etc.)

MRV: 3000 CFU/L for 100 µL swabbed in triplicate

Heat treatment is performed on all the process water samples.

Acid or combined treatments will be performed only for specific needs or special requests.

Heat treatment (on all the samples)

- Place approximately 25 mL of sample in a sterile 50-mL centrifuge tube.
- Treat with heat at 50° ± 2°C for a period of 30 ± 2 minutes.
- Continue the manipulations on the **two fractions** of the sample (treated and untreated)
- Spread undiluted 100 µL of the untreated and heat-treated fractions in **triplicate** on BCYE+BMPA agar.
- Dilute the untreated and treated fractions 1/10 with peptone water added to 0.05% of Tween 20.
- Spread 100 µL of each dilution in **duplicate** on BCYE+BMPA agar.

Acid treatment (only when necessary)

- Place 1 mL of the sample in a 2-mL tube.
- Add 1 mL of the HCl/KCl acid preparation.
- Keep in contact for 5 ± 0.5 minutes.
- Spread rapidly 200 µL in triplicate on the BCYE+BMPA agar.

Note: If 100 µL is spread, a dilution of ½ must be considered for this treatment when calculating the concentrations.

6.3.2 Drinking water samples (e.g., shower, fountains)

MRV: 10 CFU/L for 100 mL filtered

Filtration

- Place the black Millipore filter in the filtering funnel.
- Filter 10 mL of the untreated sample.
- Rinse the funnel carefully with the rinsing solution (20 – 30 mL).
- Carefully remove the membrane from the filter holder with sterile tweezers and place it (top upwards) directly on the BCYE+BMPA petri dish.
- Repeat to filter 100 mL of the untreated sample.

Be careful not to dry the filter. Stop the suction once the liquid has been completely removed. *Legionella* bacteria do not tolerate drying; they can lose their capacity for growth if they are dried.

Spreading of the samples

- Spread undiluted 100 µL of the untreated fraction in triplicate on BCYE+BMPA agar.

N.B.: If needed, the filtration of the heat-treated fraction of the sample might be done along with the untreated fraction.

6.3.3 Incubation of the samples

- Incubate at 35° ± 1°C at 2.5% CO_2 atmosphere.
- The incubation period depends on the bacterial load present in the sample. It must not be less than three days, and negative results cannot be recorded for less than 10 days of incubation.

6.4 Quantitative analysis

The quantitative analysis is performed on the spread plate that will provide the smallest MRV while allowing the enumeration, detection and isolation of *Legionella spp* colonies.

The heat-treated fraction is analyzed only when there is significant growth of heterotrophic bacteria on the untreated fraction because a loss of growth capacity on agar was demonstrated by heat treatment. A remark on the underestimation of the concentration of *Legionella spp* must be included in the analytical report.

Characteristics of the *Legionella spp* colony*:* light grey-blue colour with aspect of fritted glass by binocular loupe, can also be of variable colours: yellow, white, chestnut brown, violet, pink. The colonies can become whitish as they age. Some are fluorescent under UV lamp at 365 nm.

- Enumerate by stereomicroscope at a magnification of 60 X with a fibre optic light source each type of colony presenting the macroscopic characteristics of *Legionella.*
- Enumerate the colonies of the same type on all the Petri dishes.
- Each type of colony must be isolated in a pure culture before proceeding with confirmation.

6.5 Qualitative analysis

- Isolate each type of colony representing the phenotypical characteristics of *Legionella spp* on a BCYE agar and a TSA-blood agar; use the same colony to inoculate the two agars.

Caution: A positive bias towards types of colonies most frequently seen by the laboratory can occur over time. When several types of uncharacteristic colonies are present on the agar, an additional subculture of two or three colonies not initially targeted by the analyst must be performed.

- Incubate the BCYE at 35° ± 1°C at 2.5% CO_2 atmosphere and the TSA-blood at 35°C ± 2°C.
- If the colony grows on TSA-blood, it is essential to ensure that the same colony is a pure culture on BCYE. A mixed culture of *Legionella* and another non-fastidious bacterium could result in a false negative.
- If there is no growth on the TSA-blood, confirm with the *Instant FAME* method (refer to the MIDI® Inc. protocol).

- Proceed also with the Oxoid® latex agglutination test:
  - If the results do not allow identification with the *Instant FAME* method*;*
  - If the results have an SI less than 0.2 for *Legionella pneumophila;*
  - If the results with the *Instant FAME* method are other than for *L. pneumophila;*
  - If you need to know the serotype.

6.6 Calculation of the results

If no *Legionella* can be confirmed,

Enter < than the MRV

The smallest possible result is one colony on only one of the three Petri dishes.
Pay attention to the spreading volume.

For a positive result

- Take the number of colonies counted on the three Petri dishes.
- Calculate the number of colonies per L.
- Adjust according to the dilution or the concentration performed.

Example:

For a 100 µl spread without dilution

Petri dish: 1 = 3 confirmed colonies, 2 = 5 confirmed colonies, and 3 = 0 confirmed colonies.
Concentration in the spread volume: Sum of the colonies/spread volume
(L) = (3+5+0)/0.0003L = 26,666 CFU/L therefore, according to the significant numbers:
27,000 CFU/L.

For a spreading and a dilution of 1/10

Concentration in the spread volume (CFU/L) * (inverse of the dilution) =
27,000*(10) = 270,000 CFU/L.

For filtration of 100 mL of the sample

For a volume of 100 mL, two colonies are confirmed.
2/0.1 L = 20 CFU/L.

7. APPLICATION PARAMETERS

7.1 Limit of detection and limit of quantification

Process water: The theoretical limit of detection for the process water analytical method is 3000 CFU/L when the spread volume is 100 µL on three agars and the detection of one colony on a single Petri dish. The limit of detection is affected by the presence of heterotrophic bacteria, which can result in the necessity of diluting the sample and thus produce higher limits of detection.

Drinking water: The theoretical limit of detection for the drinking water analytical method was calculated as 10 CFU/L by considering a filtered volume of 100 mL and the detection of one colony on the Petri dish. The limit of detection is affected by the presence of heterotrophic bacteria and the particles that can reduce the filtered volume of the sample and thus result in higher limits of detection.

The limit of quantification is 1 to 300 colonies on one Petri dish.

8. RELIABILITY

8.1 Specificity of the identification method

To determine the specificity of the identification, 23 ATCC control strains of different species of *Legionella* were identified by the two qualitative analytical methods. These tests determined a specificity of 100% by genus for *Legionella*, a specificity of 100% by species for *L. pneumophila*, and a specificity of 70% for identification of the other species of *Legionella*. The difficulty determining the specificity of the other species occurs for the less common species of *Legionella* that are not included in the databases or not detected by the agglutination tests. These strains will be reported as being *Legionella* (see Appendices 1 and 2 for the lists of the different species that can be identified).

9. ACCURACY

The water samples for the validation were produced by spiking the strain of *Legionella pneumophila* ATCC 33152.

9.1 Analytical method by spread plate and dilution

Repeatability for the spread plates is 3.5%. It is obtained by analyzing four different concentrations performed six times on the same day. Reproducibility, obtained by analyzing four different concentrations by three analysts, is 5%.

9.2 Analytical method by filtration

The repeatability for the filtered samples is 6%. It is obtained by analyzing four different concentrations performed six times on the same day. Filtration volumes up to 100 mL were used for the evaluation. The reproducibility obtained by the analysis of four different concentrations by three analysts is 7%.

10. QUALITY CONTROL

10.1 Interlaboratory control

An interlaboratory quality control is carried out with Public Health of England and their FEPTU (Food and Environmental Proficiency Testing Unit) program. Twelve samples are sent to us every year. Each series contains two samples containing or not containing *Legionella*. They are sent in the form of a lenticule disc, and reconstitution in a liquid medium is performed by the laboratory.

10.2 Intra- and inter-technician control

An enumeration control is done to calculate the variation in the results for each analyst as well as the variation in the results between the different analysts. To perform this control, a sample is retained after each group of twenty enumerations to be recounted and to calculate the intra- and inter-analyst variation.

10.3 Control of the products used

A quality control is performed on 1% of the agars when they are received in order to verify their sterility.

10.4 Ongoing method performance verification during routine analysis

- A positive control is carried out with and without the performed treatment.
- A filter blank is performed at the start and after each series of filtrations.
- A spread blank is performed at the end of each series of spreads.
- For each series of analyses with the *Instant FAME* method, a check, a BLK, as well as a positive control for the method (*Legionella pneumophila*) are carried out.
- For the analyses by the agglutination test, a positive control is done for each series of analyses.

11. HEALTH AND SAFETY

11.1 Minor spill

When a small spill (a few Petri dishes) of microorganisms occurs, the technician must spread a disinfectant product (alcohol, bleach, etc.) over the contaminated surface, then cover it, and leave the laboratory for approximately thirty minutes so that the particles settle. Place a sign on the door prohibiting access to the laboratory if necessary. After thirty minutes, put on an N-95 mask before entering the laboratory, and clean the contaminated surface well. All material that came in contact with the contaminant must be treated in the autoclave.

11.2 Major spill: Wearing an N-95 mask is necessary

When there is a large spill (several Petri dishes), the technician must leave the laboratory, place a sign on the door to prohibit access, and then inform the professional in charge and his supervisor. After a 30-minute wait necessary for the particles to settle, remember to put on an N-95 mask before entering the laboratory. Pour a disinfectant product (alcohol, bleach) over the contaminated surface and cover it to allow it to act. After approximately 30 minutes, clean the contaminated surface well. All the material that came in contact with the contaminant must be placed in the autoclave. Depending on the extent of the spill, the professional in charge could require that all surfaces (walls, benches, floors, etc.) that could possibly have come in contact with the contaminant be decontaminated.

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APPENDIX 1:

List of *Legionella* species included in the MIDI *Instant FAME* system database (October 2012)

Legionella adelaidensis
Legionella anisa
Legionella birminghamensis
Legionella brunensis
Legionella cherrii
Legionella cincinnatiensis
Legionella fairfieldensis
Legionella feeleii
Legionella geestiana
Legionella gratiana
Legionella jamestowniensis
Legionella jordanis
Legionella lansingensis
Legionella londiniensis
Legionella longbeachae
Legionella nautarum
Legionella oakridgensis
Legionella parisiensis
Legionella pneumophila
Legionella quateirensis
Legionella quinlivanii
Legionella rubrilucens
Legionella sainthelensi
Legionella santicrucis
Legionella shakespearei
Legionella spiritensis
Legionella tucsonensis
Legionella wadsworthii
Legionella worsleiensis

APPENDIX 2:

List of species of *Legionella* that can be confirmed by the different OXOID agglutination tests (October 2012)

Legionella pneumophila serogroup 1
Legionella pneumophila serogroup 2-14
Legionella longbeachae 1 & 2
Legionella bozemanii 1 & 2
Legionella dumoffii
Legionella gormanii
Legionella jordanis
Legionella micdadei
Legionella anisa