3. Techniques for estimating microbial populations :

3.1. Enumeration techniques :

3.1.1. Microscopic enumeration :

These techniques offer the possibility to detect microorganisms during product control by simply examining a sample directly under an optical microscope.

It is possible to perform staining to make these microorganisms more easily visible (simple methylene blue staining and complex Gram staining).

In the case of certain liquid products (milk, yogurt…), it is necessary to dilute the sample, which will reduce the concentration of microorganisms and additional constituents.

a. Use of counting cells

Hemacytometer slides such as THOMA and MALASSEZ cells could be used for the enumeration of microorganisms.
These slides are made of glass 2 to 3 mm thick, featuring a delimited and gridded surface and covered with a cover slip so that it traps a known quantity of the food solution-dilution to be examined.

THOMA cell: large squares measuring 0.25 x 0.25 mm, with a depth of 0.1 mm, and it traps a volume of 0.1 mm³ (Figure). The cell consists of sixteen (16) large squares, each composed of 25 small squares.

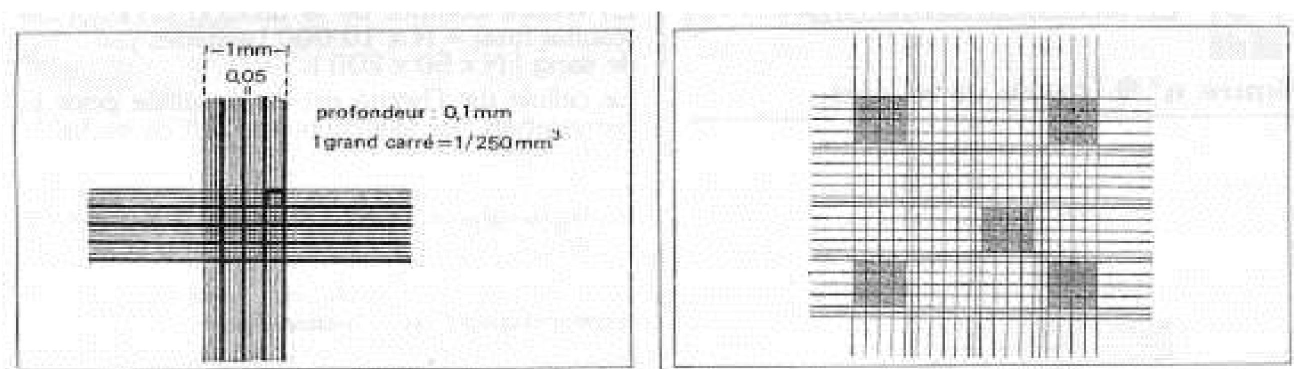


Figure: Schematic representation: Grid of the Thoma cell

- **MALASSEZ cell :** Measuring 0.2 x 0.25 mm, with a depth of 0.2 mm, and it traps a volume of 1 mm³ (Figure). The cell consists of five (5) horizontal bands, each with five (5) lines, and five (5) vertical bands, each with six (6) lines.

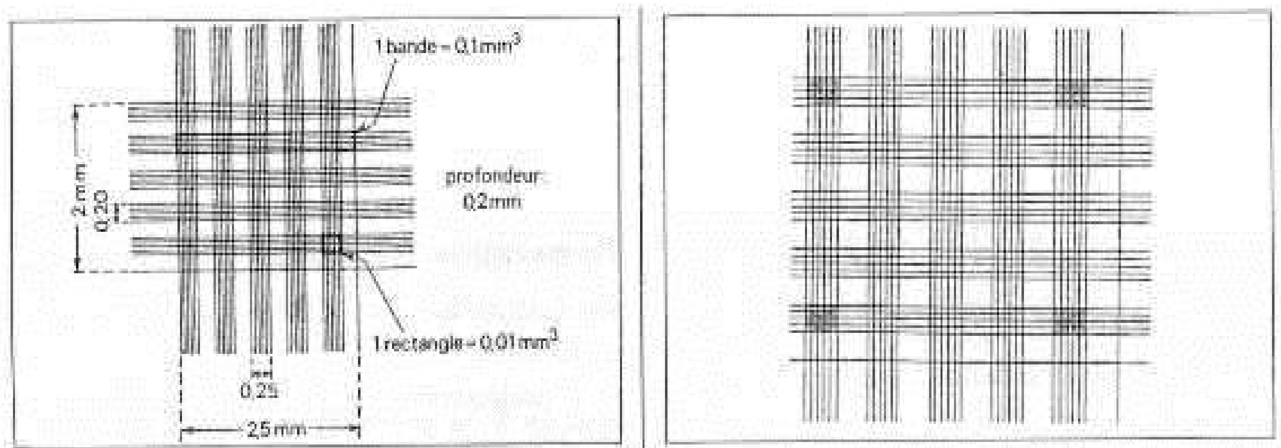


Figure: Schematic representation: Grid of the Malassez cell

bThe Breed method

This method uses slides with a delimited area of one square centimeter (1 cm²) onto which 0.01 ml of the food suspension to be examined is placed.

After drying, fixation, and staining with methylene blue or possibly Gram staining, the microorganisms are counted in 30 to 50 microscopic fields.

c. Direct epifluorescent filter technique (DEFT)

It is a microscopic enumeration technique, applied for counting microorganisms in all types of products.

Microorganisms retained on a membrane from a determined volume are counted directly under an epifluorescence microscope after staining with acridine orange. (Antibacterial activity test, heat treatment).

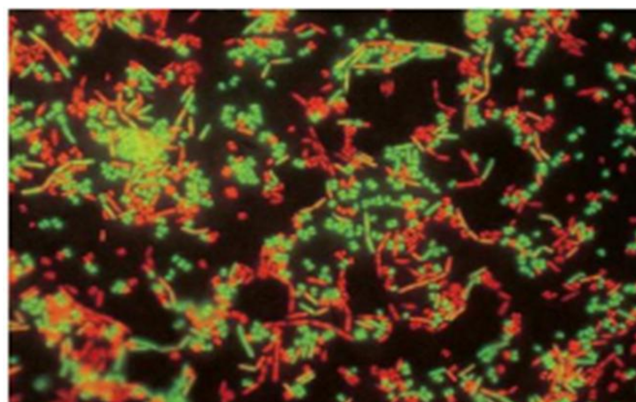


Figure: Observation under an epifluorescence microscope following staining with acridine orange.

d. Enumeration after passage through an enrichment medium

This non-quantitative technique is used for specific species (Lactobacilli, Salmonella, etc.) or when there is a correlation to be established between the number of microorganisms at the beginning and at the end of incubation.

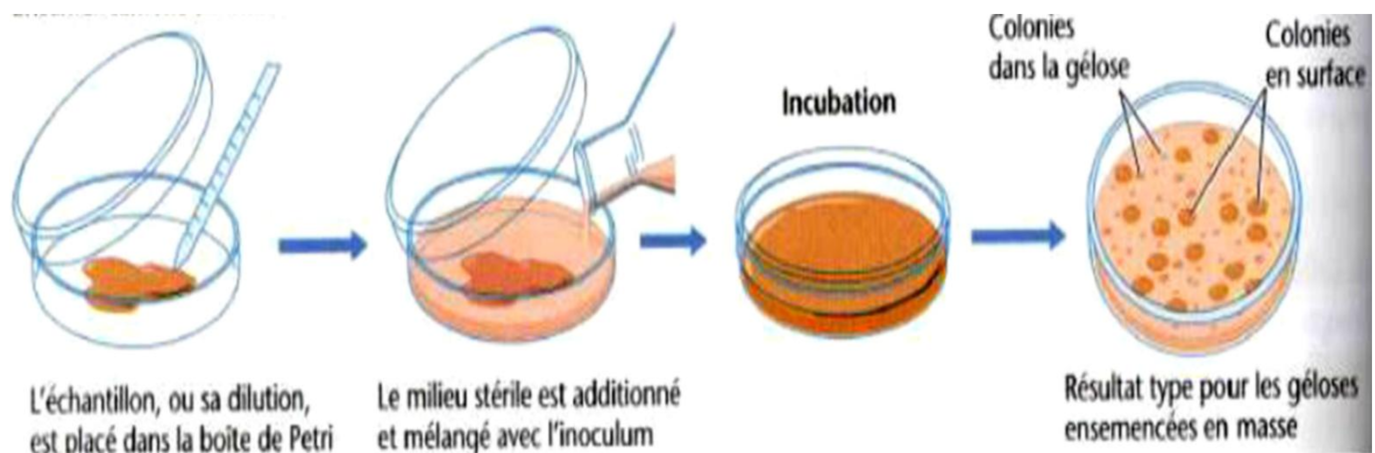
A suspension of the food to be examined in a selective enrichment medium is prepared, followed by incubation at appropriate times and temperatures for the microorganism, and then enumeration on a slide under an optical microscope.

3.1.2 Enumeration in and on solid media :

a. In agar medium (mass method)

a.1. Plate Count Agar (Standard Methods Agar

This is the standard method for enumerating aerobic germs. Its use requires a clear growth medium to allow colony counting using a colony counter.



a.2. Double layer technique

Anaerobic germs require a second layer of agar to maintain an anaerobic environment. This technique is used to prevent the overgrowth of colonies on the surface. First, three-quarters of the agar medium is poured. Inoculation is performed after the medium solidifies. The remaining quarter, kept in a supercooled state in a water bath, is poured over the first layer.

a.3. « ROLL TUBE COUNT » technique

As the name suggests, this technique involves the use of 25 mL screw-cap tubes. 2 to 4 mL of agar medium is poured into these tubes, which are then kept in a super-cooled state (45°C) in a water bath.

0.1 mL of each dilution is added to the tubes, which are then rolled horizontally under cold water until the medium forms a uniform layer on the walls.

It was proposed for the culture of strict aerobic bacteria and Koch's bacilli (tuberculosis bacteria), which are difficult to grow.

The medium used is transparent, allowing for easy colony counting.

a.4. Agar droplet technique

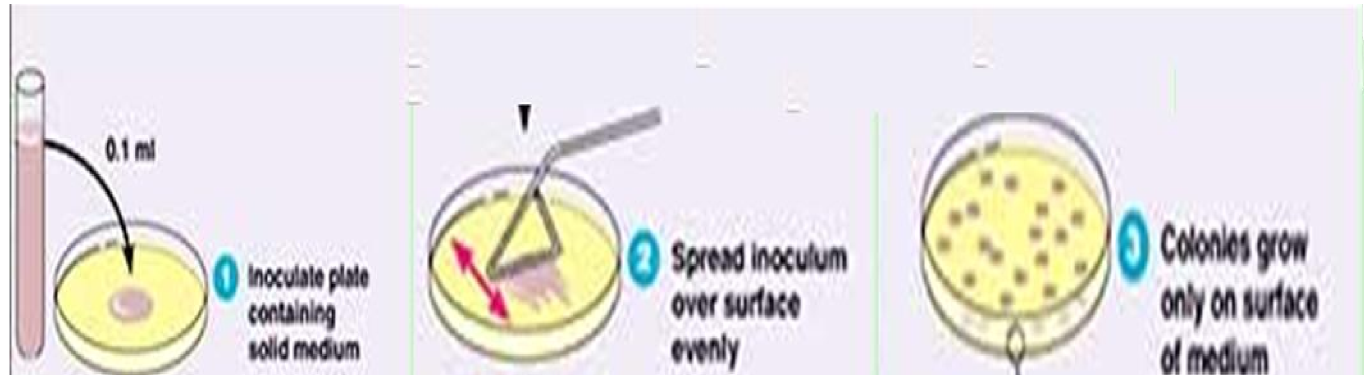
This technique is proposed in a miniaturized and cost-effective format.It involves mixing a diluted homogenate of any food product into a melted and cooled (45°C) agar medium, which is then distributed in 0.1 mL droplets onto the bottom of a Petri dish.

It is therefore possible to place 5 droplets from 4 successive dilutions in the same plate.After incubation, a special projector enlarges the diameter of the droplets, allowing for the counting of pinpoint colonies.

b. Surface spreading (spread plate)

This method is preferable when selective media are used for the enumeration of a specific group of aerobic microorganisms.

It allows for the manifestation of colonial properties of these microorganisms, such as: morphology, pigmentation, hemolysis, precipitation halos, or color changes in the culture medium.



c. Membrane filtration

This technique is used to estimate the number of microorganisms during the control of food liquids (water, beverages...). It involves concentrating microorganisms on a membrane using a single or multi-position filtration device.

The filters used are made from:

- a mixture of cellulose esters.;
- similar polymers (cellulose nitrate, cellulose acetate);
- other polymers: polyamide, polypropylene, polycarbonate, polytetrafluoroethylene (PTFE).

The number of colonies N per ml of sample is calculated as follows:

N = n / v, where n is the number of colonies, and v is the sample volume, which is usually equal to 100 ml.

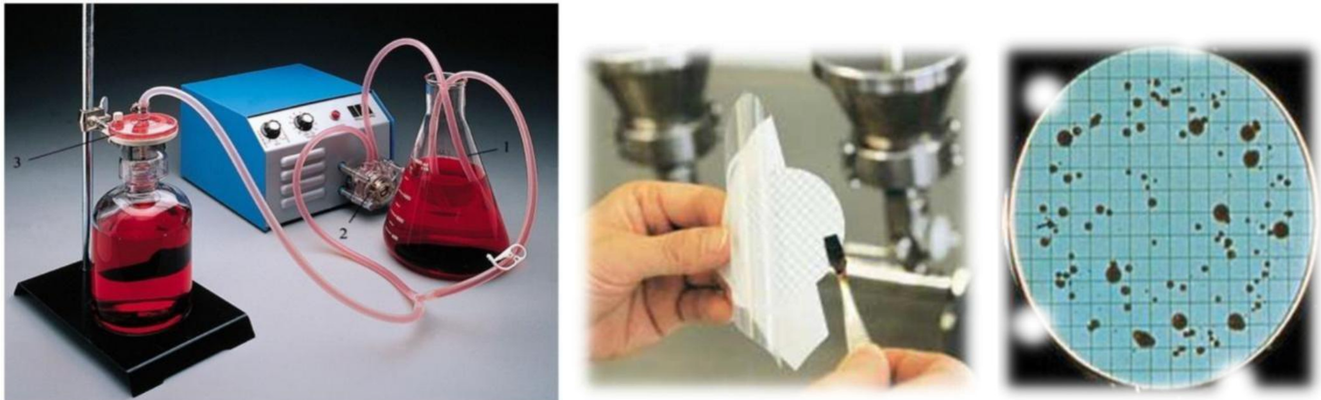


Figure: Membrane filtration setup, Membrane filter after culture.

d. Dip slids

During the inspection of food products and/or surfaces, slides and dip slides are used to estimate the number of microorganisms.

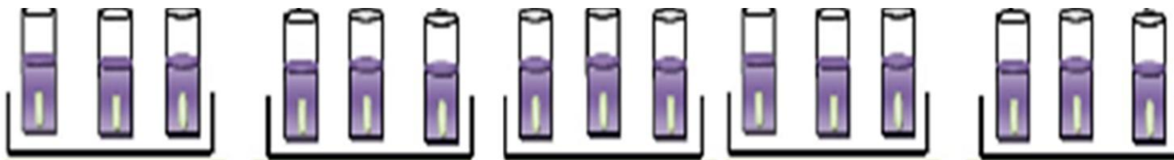
Figure : (a)Contrôle du nettoyage et de la désinfection (b) bandes à immerger après culture

3.1.3. Liquid medium enumeration:

This liquid medium enumeration method is known as the Most Probable Number (MPN) method by McGrady. a statistical technique used in microbiology (especially water/food testing) to estimate microbial density from serial dilutions, where the pattern of positive (growth/gas) tubes (e.g., 3-2-1) in sets of three (or more) is matched to a standard table, often referencing pioneers like M.H. McCrady, to find the estimated bacterial count.

It is commonly used for the detection and enumeration of total coliforms, fecal coliforms, and fecal streptococci in water.

This technique is based on the use of series of tubes containing liquid medium.



Example of enumeration by MPN (Most Probable Number): Enumeration of total and fecal coliforms (colimetry)

- **Coliforms:**

They are enterobacteria belonging to different genera: *Citrobacter*, *Enterobacter*, *Escherichia*..., frequently found in the environment as well as in the intestines of mammals, including humans. They ferment lactose with gas production at 30°C.

Coliforms are Gram-negative bacilli (GNB), non-spore-forming, oxidase-negative, facultative anaerobes, capable of growing in the presence of bile salts, and capable of fermenting lactose with acid and gas production within 24 to 48 hours at 37°C.

- **Thermotolerant (Fecal) Coliforms:** (Indicator of fecal contamination) Coliforms that ferment lactose with gas production also at 44°C;

- **E. coli:** Thermotolerant coliform that produces indole at 44°C.

****Detection and Enumeration of Coliforms:****

In water, coliform bacteria are tasteless, odorless, and colorless; moreover, they can only be detected through laboratory analysis.

According to the Canadian Drinking Water Quality Guidelines, the maximum acceptable concentration of total coliforms in drinking water has been set at "**no detectable microorganisms per 100 ml volume**."

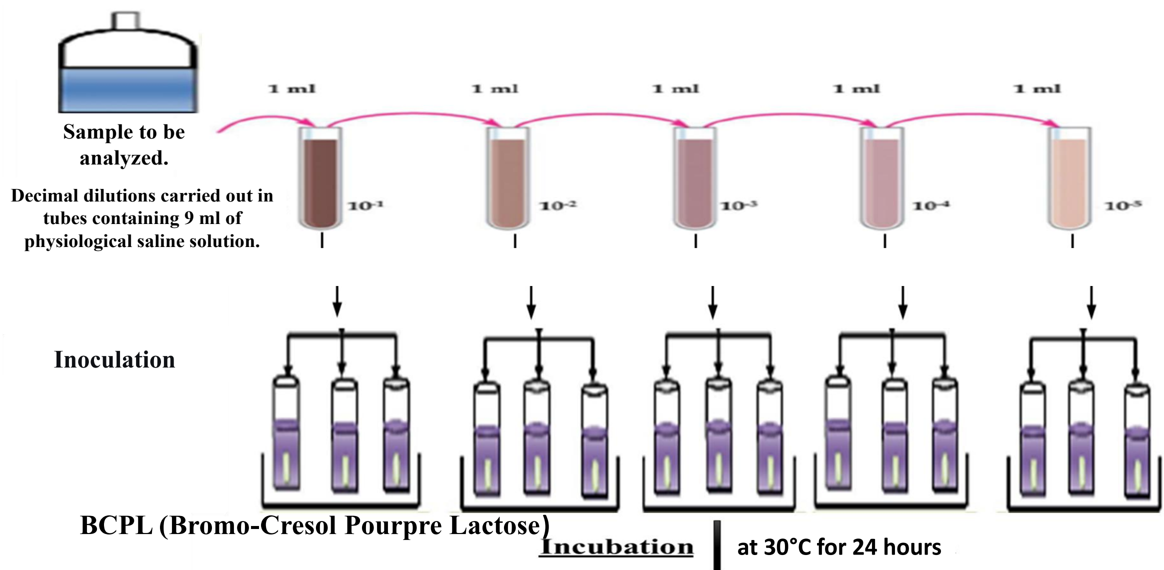
****Principle****

Coliform bacteria have the specific property of fermenting lactose into lactic acid and releasing gas, thereby acidifying the culture medium and causing a change in the pH indicator. Additionally, gas production becomes visible in inverted Durham tubes.

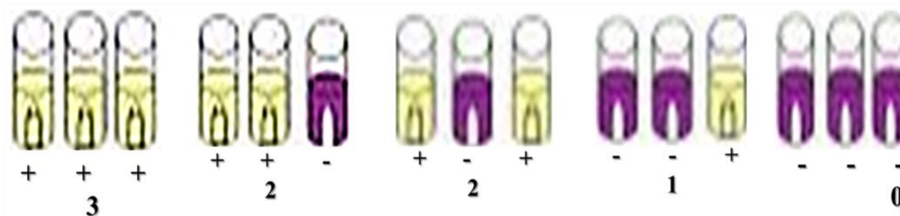
Procedure

1. **Presumptive Step:** Reserved for the detection of total coliforms.

From the water sample to be analyzed, inoculate 15 tubes containing BCPL medium:



Change of the medium from violet to yellow + gas in the Durham tubes = presence of total coliforms.



After incubation, the number of positive tubes in each series of three is counted, and the characteristic three-digit number is determined.

Interpretation

Test tubes are considered positive if they exhibit both:

- Gas evolution
- Microbial growth accompanied by a yellow discolouration of the medium (which indicates the fermentation of the lactose present in the medium).

⑤ Lire la valeur du NPP dans la table de Mac Grady et en déduire la concentration des bactéries dans l'échantillon :

Nombre de tubes positifs au niveau des 3 taux de dilution retenus	NPP	Nombre de tubes positifs au niveau des 3 taux de dilution retenus	NPP
000	< 0,3	230	2,9
001	0,3	300	2,3
010	0,3	301	4
020	0,6	302	6
100	0,4	310	4
101	0,7	311	7
110	0,7	322	12
111	1,1	320	9
120	1,1	321	15
121	1,5	322	21
200	0,9	323	29
201	1,4	330	20
210	1,5	331	50
211	2,0	332	110
220	2,1	333	>110
221	2,8		

Therefore :

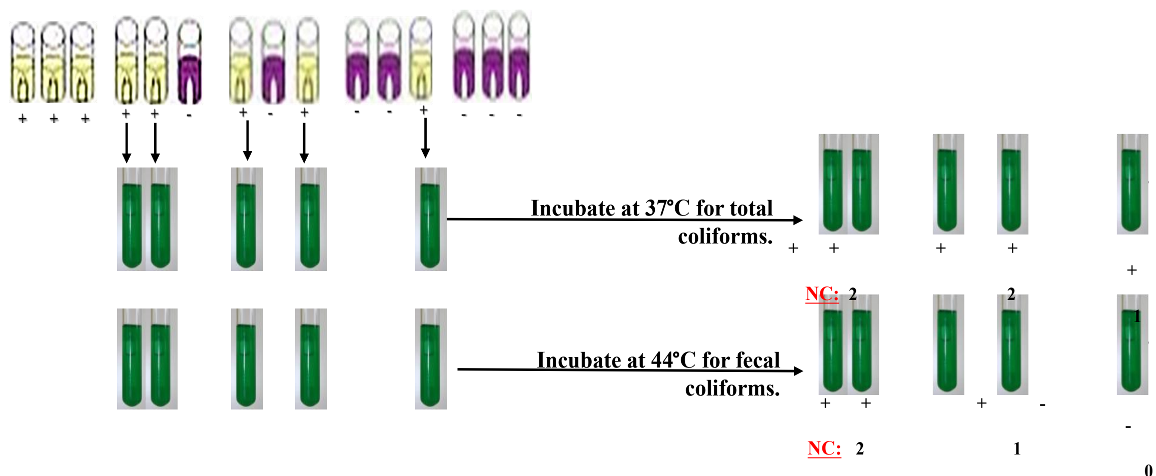
$$C\ bacteria = \frac{n}{\text{(dilution factor corresponding to the first digit)}}$$

2. Confirmatory step

The confirmatory test is based on the detection of faecal coliforms (thermotolerant), among which the presence of Escherichia coli is of particular concern.

Thermotolerant coliforms have the same fermentation properties as coliforms, but at 44°C.

- Based on the positive results from the preliminary stage, re-inoculate a tube of BLBVB medium (**Bright Green Lactose-Bile Broth**) with 1 ml from each positive tube.



3.2. Biomass quantity estimation techniques :

Evaluating microbial activity is as important as evaluating the number of microorganisms.

3.2.1 Spectroscopic

a. Dyes reduction (Colourant reduction)

The principle is based on the response of a redox dye to the presence of metabolically active microorganisms, which results in a color change.

Two dyes are commonly used to estimate the number of viable microorganisms: methylene blue (which changes from blue to colorless) and resazurin, which has been used in the testing of milk and fresh or minced meat. This dye is reduced and changes from blue to pink to colorless.

The time required for decolorization can be measured to assess the number of viable microorganisms. The assessment of decolorization is usually done visually, by observation under a microscope, or using a spectrophotometer.

b. Enzymatic activity measurement

The redox dye technique is based on the evaluation of reductase activity. However, many other enzymes have been used to detect and assess the presence of microorganisms, such as: phosphatases, esterases (used to estimate the number of viable bacteria in milk, meat, and fish), and glutamate decarboxylase (used to estimate the number of *E. coli* in milk).

c. Radiometric markers

This technique is based on the incorporation of ^{14}C (carbon-14) into a growth medium, so that when microorganisms utilize this metabolite, $^{14}CO_2$ is released and subsequently measured using a radioactivity counter or a spectrophotometer.

^{14}C is incorporated as ^{14}C-glucose for microorganisms that typically use it; otherwise, it is incorporated as ^{14}C-formate or ^{14}C-glutamate for others.

The technique involves culturing the microorganisms in a medium containing the labeled molecule. After incubation, the culture is periodically tested to detect the presence of $^{14}CO_2$.

The time required to detect $^{14}CO_2$ is inversely proportional to the number of microorganisms present.

3.2.2. Electrochemical :

a. Impedancemetry

Cette technique mesure la baisse de l'impédance dans un milieu pourvu de microorganismes. Over time, the microorganisms present in the medium degrade large, electrically neutral or weakly charged molecules (proteins, polysaccharides, etc.) and produce smaller, ionized molecules (amino acids, organic acids, etc.), leading to a decrease in the impedance of the medium.

The technique involves culturing the microorganisms in cuvettes, at the bottom of which the measuring electrodes of the bactometer are fixed.

It is used for the detection and quantification of major contaminants (aerobic bacteria, Enterobacteriaceae, coliforms, lactic acid bacteria, yeasts, and molds).

3.2.3. Other Processes (Microcalorimetry)

This technique is based on the measurement of slight heat variations (measurement of the enthalpy involved in the degradation of growth substrates). The heat production, measured using microcalorimeters, is closely linked to the catabolic activities of microorganisms.