Chapter 2: Microbial Inactivation

Introduction

Microbial inactivation is a cornerstone concept in numerous scientific and industrial fields, including food preservation and safety, pharmaceutical sterilization, water treatment, and clinical disinfection. The primary goal is to reduce or eliminate the microbial population in a product or environment to prevent spoilage and disease transmission. To achieve this, we must answer fundamental questions: How do we scientifically distinguish a living microorganism from a dead one? How can we measure the effectiveness of a lethal agent (such as heat, UV radiation, or a chemical disinfectant)? This chapter provides a detailed exploration of these concepts, the methods used to quantify viability, and how to construct and interpret microbial survival curves.

1.1 Defining Viability in Microorganisms

Defining "life" and "death" for a single-celled organism is more operational than philosophical. A cell can be in various states, making accurate measurement a challenge.

Cell viability can be defined as the number of living cells in a cell population.

- **Viable (Living) Cell:** The gold standard definition in classical microbiology is a cell that is capable of **autonomous reproduction** under favorable conditions. This means it can undergo binary fission to form a visible colony. This ability is termed **culturability**.
- **Non-Viable (Dead) Cell:** A cell that has **irreversibly lost its ability to reproduce**. It's important to note that a dead cell might still possess transient characteristics of living cells, such as an intact cell membrane (until lysis occurs) or residual enzymatic activity. This ambiguity is why multiple detection methods exist.
- **Viable But Non-Culturable (VBNC) State:** This is a critical concept to understand. When exposed to sublethal stress (e.g., low temperature, starvation, osmotic shock), many bacteria (like *Vibrio cholerae* or *Legionella pneumophila*) can enter a dormant state. In this state, they **fail to grow on standard culture media** (so they are scored as "dead" by plate counting methods) but remain **metabolically active and alive**. Under specific, favorable conditions, they can "resuscitate" and regain culturability. This has significant implications for public health, as VBNC pathogens can be undetected by routine testing but still pose a risk.

1.2 Methods for Detecting Viability

Methods are broadly categorized into those that **measure reproductive capacity (culture-based)** and those that measure **cellular functions or structures (alternative viability indicators).**

Method	Principle	What it Measures	Advantages	Disadvantages & Limitations
Culture-Based Methods (Plate Counts)	A diluted microbial suspension is spread or poured onto a nutrient agar medium. After incubation, each viable and culturable cell grows into a visible colony. Colonies are counted and reported as Colony Forming Units (CFU).	**Reproductive capacity (Culturability).** This is the definitive proof of a cell's ability to multiply.	- **Sensitivity:** Can detect a single viable cell in a sample. - **Reference Method:** It is the historical and industry-standard method. - **Isolation:** Allows for the isolation and further identification of the surviving microorganism.	- **Time-Consuming:** Results take 24 to 48 hours or even weeks for slow-growing organisms. - **VBNC Blindness:** Fails to detect cells in the VBNC state, leading to an underestimation of the true number of living cells. - **Medium Bias:** The choice of culture medium, temperature, and atmosphere can selectively inhibit some viable cells.
Viability Indicators (Alternative Methods)	These techniques use specific markers (often fluorescent dyes or probes) to assess key cellular functions or structures indicative of life, without requiring growth.	**Membrane integrity or a specific metabolic activity.** These are proxies for viability.	- **Speed:** Results are obtained in minutes (e.g., via flow cytometry or fluorescence microscopy). - **VBNC Detection:** Can detect cells in the VBNC state. - **High-Throughput:** Easily automatable for analyzing many samples quickly.	- **Indirect Measurement:** Does not prove reproductive ability. A cell with an intact membrane may still be dead. - **Cost:** Requires specialized and expensive equipment (flow cytometer, epifluorescence microscope). - **Subjectivity:** Setting the threshold between "live" and "dead" based on fluorescence intensity can be subjective.

Culture-Based Methods

Étalement sur boîte

L'échantillon, ou sa dilution, est placé à la surface du milieu gélosé (0,1 mL ou moins)

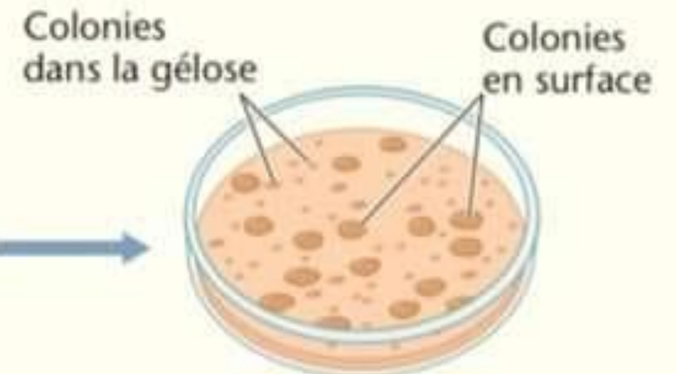
L'inoculum est étalé sur l'ensemble de la surface de la boîte de Petri à l'aide d'un râteau de verre

Résultat type pour les géloses ensemencées par étalement

Ensemencement en masse

L'échantillon, ou sa dilution, est placé dans la boîte de Petri

Le milieu stérile est additionné et mélangé avec l'inoculum



Résultat type pour les géloses ensemencées en masse

Ensemencement manuel

dilution 1/10e — dilution 1/10e — dilution 1/10e — dilution 1/10e

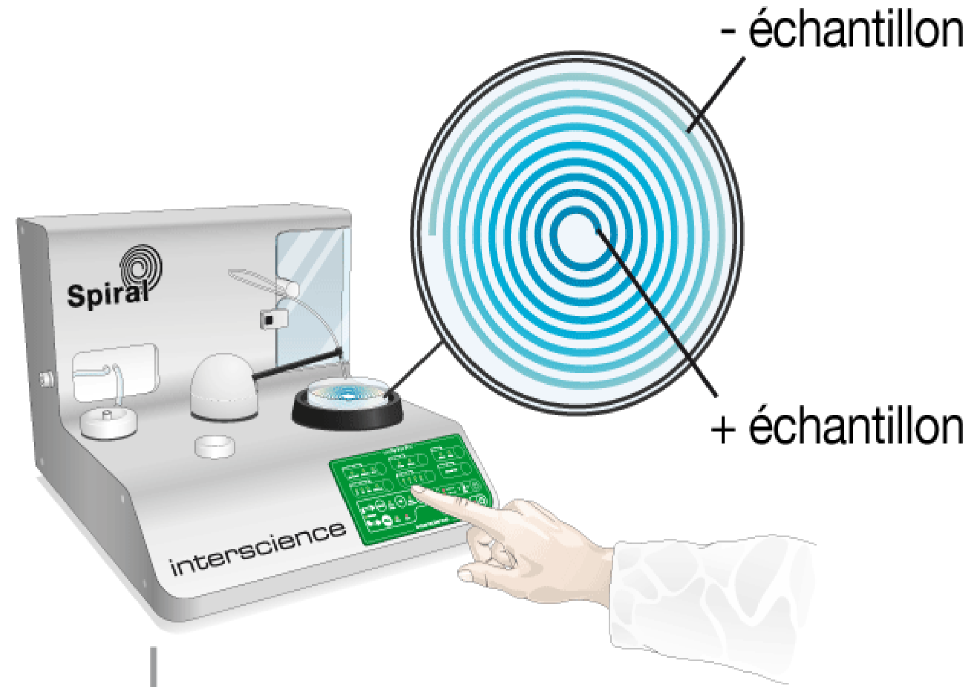
1/10 — 1/100 — 1/1000 — 1/10000

OK

UFC > 300 : non exploitable

UFC < 10 : non exploitable

Ensemencement Spiral



OK

- De **100 à $1x10^7$ UFC/mL** sur 1 seule boite de Petri
- **Jusqu'à 75 % d'économies en consommables**
- Cycle complet en **25 secondes** !

Manual cell counting using a Malassez chamber

Tiefe
Depth
Profondeur
0,200 mm
0,0025 mm²
Malassez
MARIENFELD

a Malassez chamber

0,200 mm
Malassez
10 rectangles
Rectangle non quadrillé
Rectangle quadrillé verticalement
Rectangle quadrillé horizontalement
10 rectangles
Rectangle subdivisé en 20 petits carrés

Exemple de lame en verre avec son quadrillage

Schéma d'une portion de la lamelle
10 rectangles
10 rectangles
1 rectangle = 1µl/100 = 0,01 µl
1 petit carreau = 1/20 de rectangle

Viability Indicators:

Cell viability assays aim to measure activities related to cell maintenance and survival.

Typically, metabolic biomarkers such as ATP or enzymatic activity are monitored through dye inclusion tests by living cells.

A number of these assays determine the number of living cells by means of the metabolization of colored substrates by metabolically active cells, or through the accumulation of vital dyes. As these metabolic activities or dye accumulation are specific to living cells, they are considered to be directly proportional to the number of viable cells.

✓ Performed in multi-well plates, which allows for rapid, large-scale measurements.

✓ Based on the use of dyes or fluorochromes; cell viability is assessed by measuring absorbance, comparing control cultures to cultures with different culture conditions.

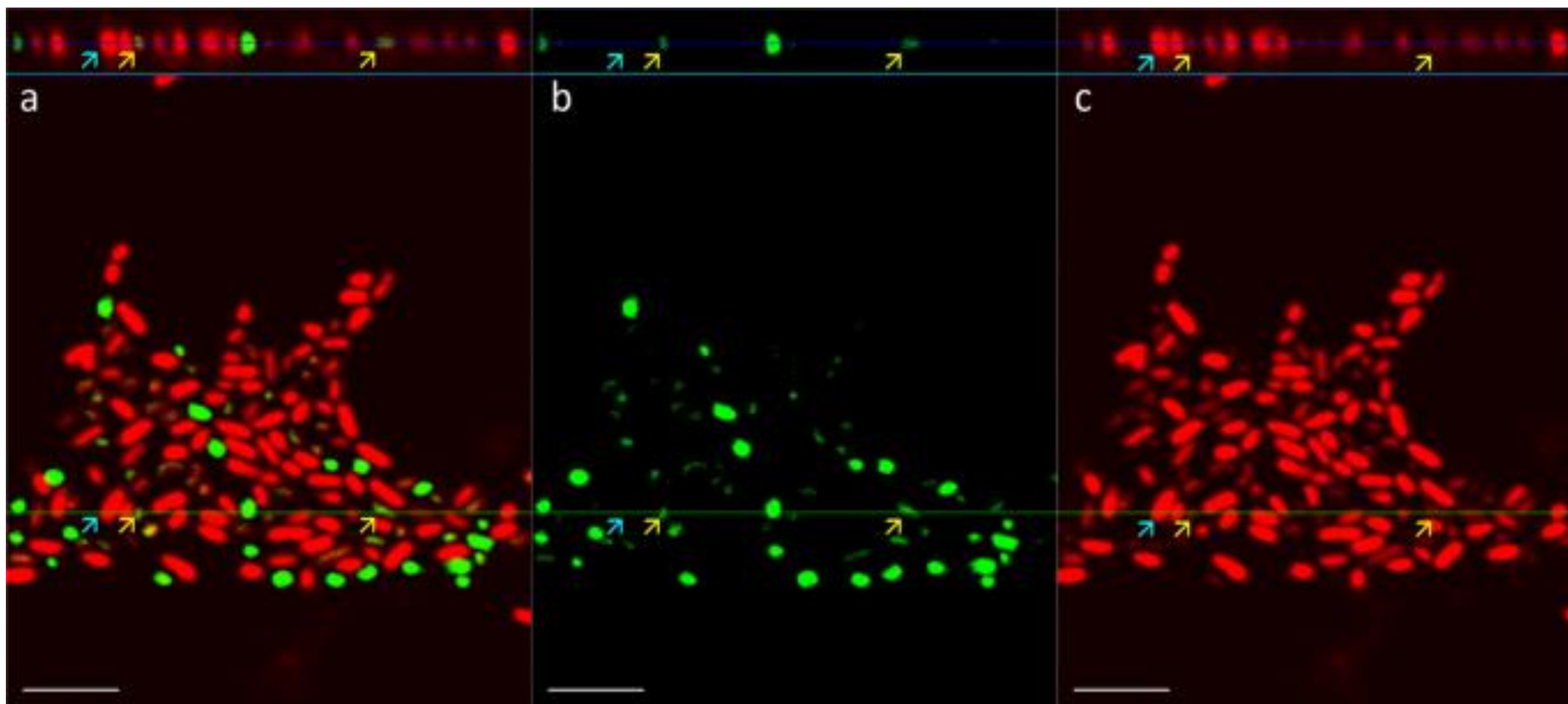
1.Membrane Integrity : This is a rapid test based on the principle that an intact cytoplasmic membrane is essential for life.

A mixture of two nucleic acid-binding dyes is used:

1. **SYTO 9 (Green Fluorescence):** A membrane-permeant dye. It enters **all** cells, both those with intact and damaged membranes, and stains their DNA/RNA.
2. **Propidium Iodide (PI) (Red Fluorescence):** A membrane-impermeant dye. It **only** enters cells with severely compromised cytoplasmic membranes. PI has a higher binding affinity for nucleic acids than SYTO 9 and will displace it.

- **Result :** Under a fluorescence microscope or in a flow cytometer:

1. **Cells with intact membranes** exclude PI, so SYTO 9 staining makes them appear **green**. These are scored as "live."
2. **Cells with damaged membranes** allow PI to enter. PI binds to the nucleic acids, overriding the SYTO 9 signal, making them appear **red**. These are scored as "dead.“



Confocal laser scanning microscopy (CSLM) images of 24 h *E. coli* biofilm co-stained with propidium iodide (PI) and SYTO 9: vertical and horizontal cross-sections in multichannel (**a**), green channel (**b**) and red channel (**c**) view. Dead cells stained with PI are indicated with cyan and viable cells double-stained with PI and SYTO 9 with yellow arrows.

2. Metabolic Activity (Resazurin Reduction Test):
This test measures the metabolic activity of a cell population.

- Resazurin is a non-fluorescent, blue dye that is cell-permeable. Metabolically active cells, through their reducing power (from enzymes like NADH dehydrogenases in the electron transport chain), reduce resazurin irreversibly to **resorufin**. Resorufin is highly fluorescent and pink in color.

- The intensity of the fluorescence or the color change (from blue to pink) is directly proportional to the number of metabolically active cells in the sample. A non-fluorescent, blue sample indicates a lack of metabolic activity.

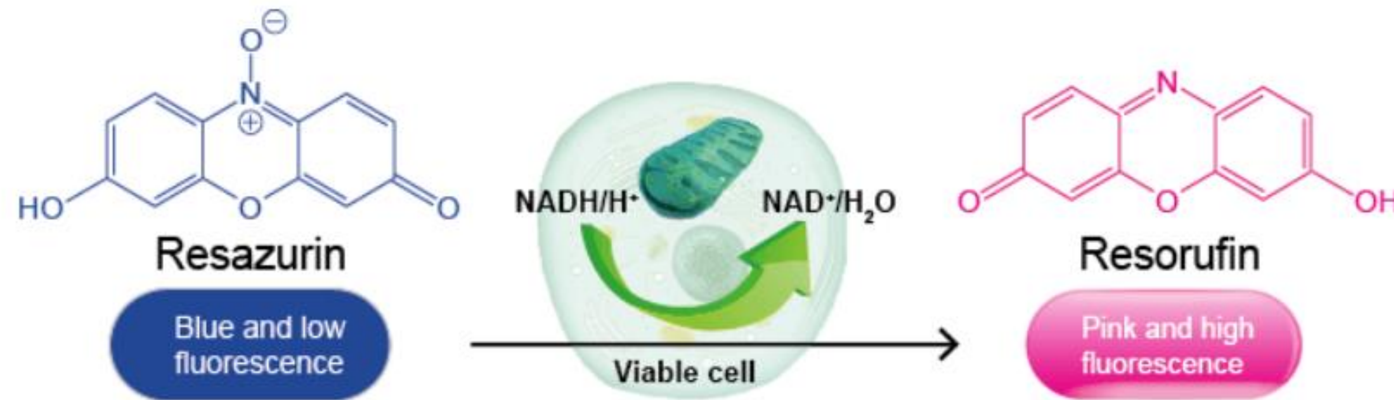


Figure 1. The principle of resazurin cell viability assay.

Obtaining survival curves using plate counting techniques

When a lethal treatment is applied to a microbial population, the number of viable cells decreases over time. A **survival curve** is the graphical representation of this decline, and it is a fundamental tool in quantitative microbiology.

2.1 Common plating methods

To determine the number of survivors at a given time point, a known volume of the diluted sample must be plated. The choice of method can impact the result.

1.Spread plate method:

A small volume (typically 0.1 mL) of a diluted sample is pipetted onto the surface of a pre-poured and dried agar plate. A sterile bent glass rod (hockey stick) or disposable spreader is used to distribute the inoculum evenly over the entire surface.

2. Pour plate method:

A larger volume (typically 1 mL) of a diluted sample is pipetted into a sterile, empty Petri dish. Molten nutrient agar, cooled to a temperature of 45-50°C (just above its solidification point), is then poured into the dish. The dish is gently swirled in a figure-eight pattern to mix the inoculum thoroughly with the agar before it solidifies.

2.2. Calculations: Number, Fraction, and Percentage of Survivors

After incubation, plates with 30-300 colonies are selected for counting, as this range provides statistically reliable data. The result is expressed in **Colony Forming Units (CFU)** .

1.Calculating the concentration of survivors (N):

N (CFU/mL) = (Number of colonies counted) / (Volume plated in mL) x Dilution factor

Example: A milk sample is heat-treated. At time *t*, 0.1 mL of the 10^{-5} dilution is spread-plated. After incubation, 65 colonies are counted on the plate.

- N = 65 colonies / (0.1 mL) x 10^5 = 65 x 10 x 10^5 = 65 x 10^6 = **6.5 x 10^7 CFU/mL**.

2.Calculating the fraction of survivors (S):

This dimensionless number compares the population at a given time (Nt) to the initial population (N0).

$$S = Nt / N0$$

Example: Initial count N0= 2.0 x 10^8 CFU/mL. After 3 minutes of treatment, N(3min) = 6.5 x 10^7 CFU/mL.

- S = (6.5 x 10^7) / (2.0 x 10^8) = **0.325**

3.Calculating the percentage of survivors (% S):

This expresses the survivor fraction as a more intuitive percentage.

$$\% S = (Nt / N0) \times 100$$

Example: % S = 0.325 x 100 = **32.5%**

2.3 Practical examples and essential laboratory precautions for survival curves

Example : Thermal inactivation of *Listeria monocytogenes* at 65°C

Time (min)	Dilution Plated	Colonies Counted (for 0.1 mL spread plate)	N (CFU/mL)	logN
0	10^{-6}	220	2.2×10^{9}	9.34
1	10^{-6}	115	1.15×10^{9}	9.06
2	10^{-5}	250	2.5×10^{8}	8.40
3	10^{-4}	180	1.8×10^{7}	7.26
4	10^{-3}	95	9.5×10^{5}	5.98
5	10^{-2}	42	4.2×10^{4}	4.62

Constructing the survival curve:
The data is plotted with the **log of the survivor count (log N= f(t)**) on the **Y-axis** versus **treatment time** on the **X-axis**. This is often done on semi-logarithmic graph paper or using software.

Critical laboratory precautions for accurate survival curves:

1.Mastering dilution technique: This is the single greatest source of experimental error.

1. Use calibrated micropipettes with sterile, aerosol-barrier tips.
2. Always use fresh, sterile diluent (e.g., 0.1% peptone water or saline) to maintain osmotic balance and prevent cell death or growth during the procedure.
3. **Vortex or mix each dilution tube thoroughly for at least 5-10 seconds** immediately before pipetting from it. Cells sediment quickly; without proper mixing, the plated aliquot will not be representative of the tube's concentration.

2.The "30-300" colony counting rule:

1. Plates with >300 colonies are considered "Too Numerous To Count" (TNTC). Overcrowding leads to nutrient depletion, competition, and the merging of colonies, resulting in an inaccurate, underestimated count.
2. Plates with <30 colonies have a high statistical error margin. A difference of just a few colonies leads to a large variation in the calculated CFU/mL.
3. *Principle:* **Only count plates falling within the 30-300 colony range for reliable, quantitative data.**

1.The Problem of Cell Aggregation (Clumps and Chains):

1. A chain of 4 streptococci cells or a clump of staphylococci will only give rise to a **single colony**. Therefore, the CFU count always represents the number of colony-forming *units* (which could be single cells or clusters), not the absolute number of individual cells. This causes an inherent underestimation.
2. *Mitigation:* Ensure proper dispersion before and during dilution. This can be aided by using diluents containing low concentrations of surfactants (like Tween 80) or by using mechanical disruption (vortexing with glass beads) for clump-forming organisms.

2.Neutralization of the Lethal Agent (Carry-Over Effect):

1. When a sample is taken from a treatment vessel (e.g., a tube containing a disinfectant), it still contains the active lethal agent. If this small volume is transferred directly into the first dilution tube or onto the plate, the inactivation process continues, artificially lowering the survivor count.
2. *Mitigation:* The lethal agent must be neutralized immediately upon sampling.
   1. **Physical Neutralization:** For heat, use **ice-cold** diluent for the first dilution to rapidly lower the temperature and halt thermal death.
   2. **Chemical Neutralization:** For disinfectants, use a diluent containing a specific neutralizer (e.g., for chlorine or peroxide, use diluent with sodium thiosulfate; for quaternary ammonium compounds, use diluent with Lecithin and Tween 80).

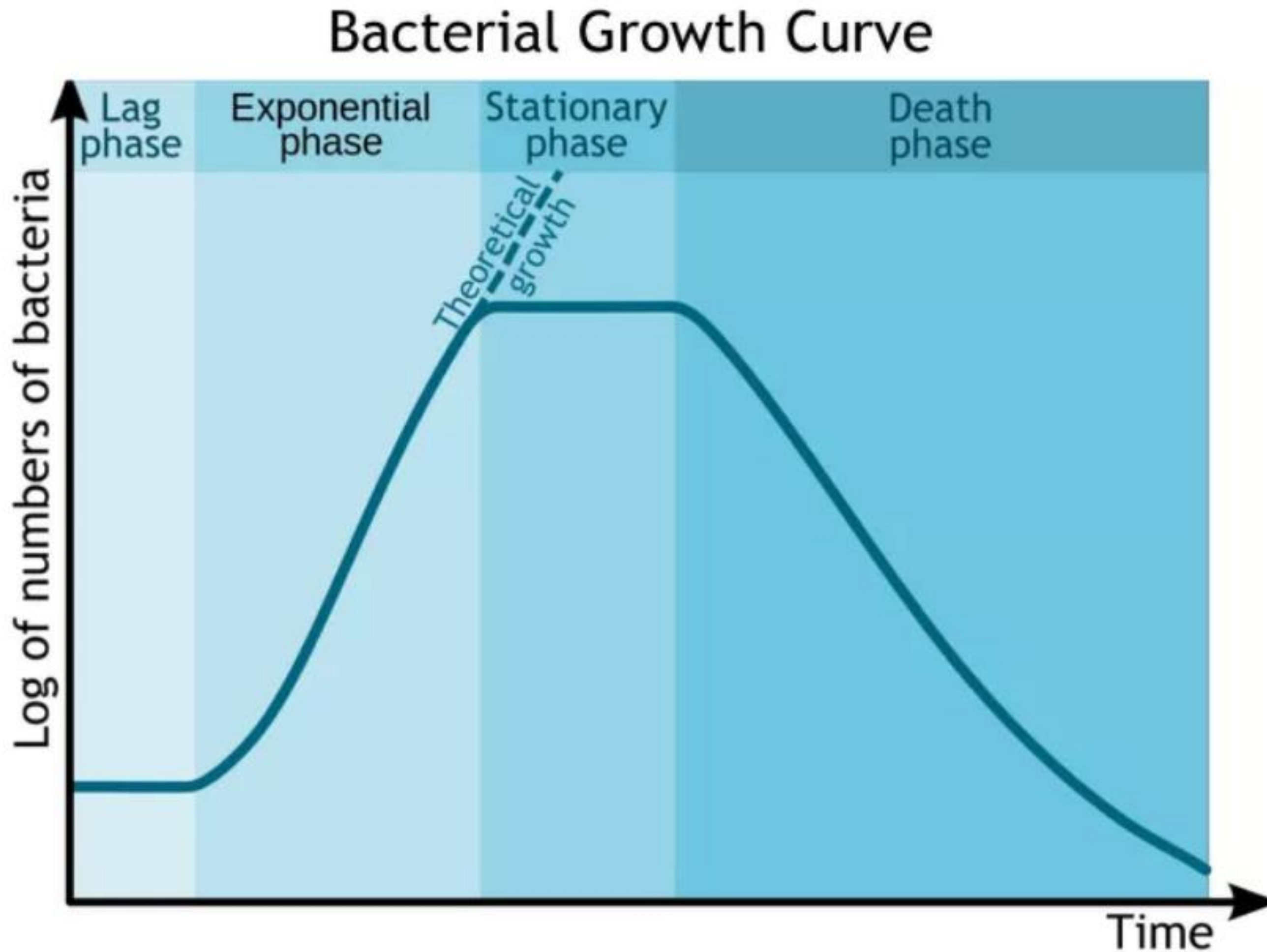
3.Ensuring Reproducibility:

1. For each time point and each dilution, always plate in **duplicate or triplicate**. This provides a measure of the random error associated with pipetting and plating.
2. Calculate the **mean and standard deviation** of the colony counts. Plotting error bars on the survival curve provides a visual representation of the data's reliability.

THERMAL DESTRUCTION OF MICROORGANISMS

I. Review of growth and factors influencing microorganism development

Under favorable conditions, microorganisms have a remarkable growth rate. In general, the reproduction of a germ takes 10 to 30 minutes. When a medium is inoculated, growth occurs in 4 schematic phases:



Parameters of microbial growth

1.Growth rate

The growth rate μ (h^{-1}) measures the increase in the microbial population over a given period of time t and under specific conditions, as follows:

$$\frac{dX}{dt} = \mu X \Rightarrow X = \frac{1}{\mu}\frac{dX}{dt}$$

X represents the biomass (expressed in g/L or in number of cells)

Note: The maximum growth rate (μmax) is calculated during the exponential phase by projection onto the graph axes (μmax = tg).

2.Generation time or doubling time

This time G (h) is given by: **G = ln2 / μmax**

3.Number of generations

At time t of fermentation, the number of generations N is: **N = t / G**

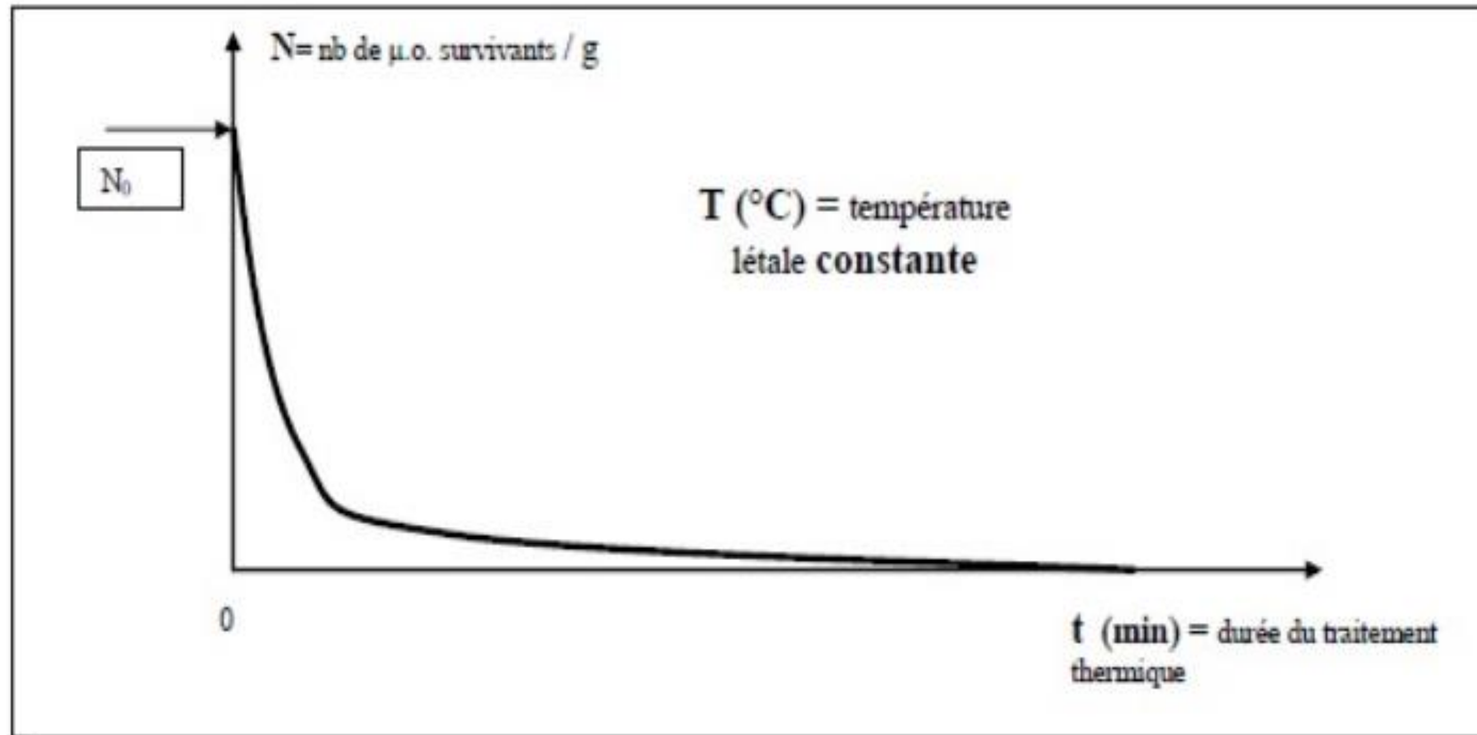
Kinetics of microorganism destruction

Influence of heat treatment duration

1- The survival curve

At different times, the number of surviving microorganisms is determined following exposure to a constant lethal temperature.

The following figure shows the shape of the curve: **N=f(t)**



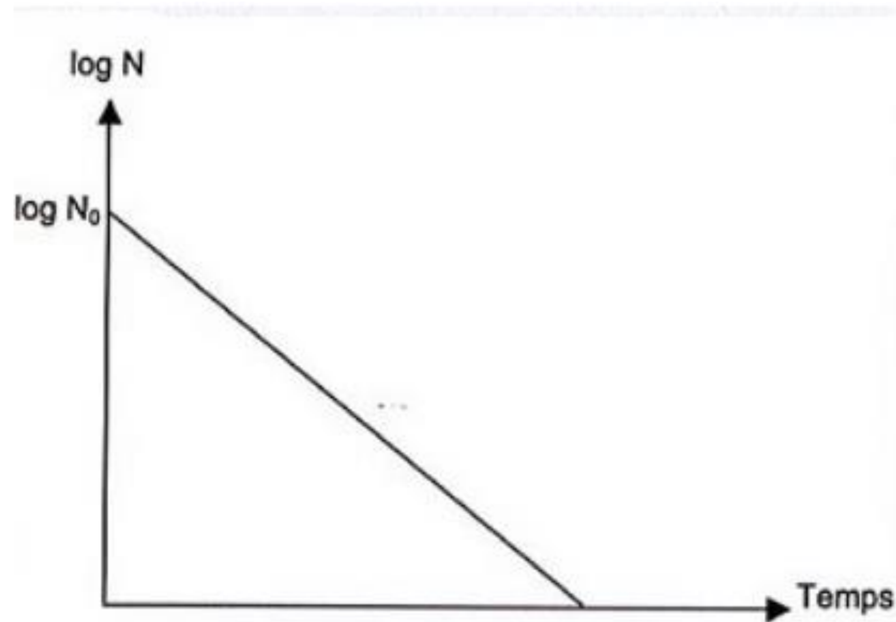
Legend:
N0 = initial microorganism concentration
N = concentration of surviving microorganisms
t = duration of heat treatment

We obtain an exponential decay curve: this is the survival curve. It shows that the microorganisms are destroyed progressively.

In order to determine the equation of the curve, the previous curve is plotted on a semi-logarithmic scale, i.e.:

Log(N) = f(t)

The following curve is obtained:

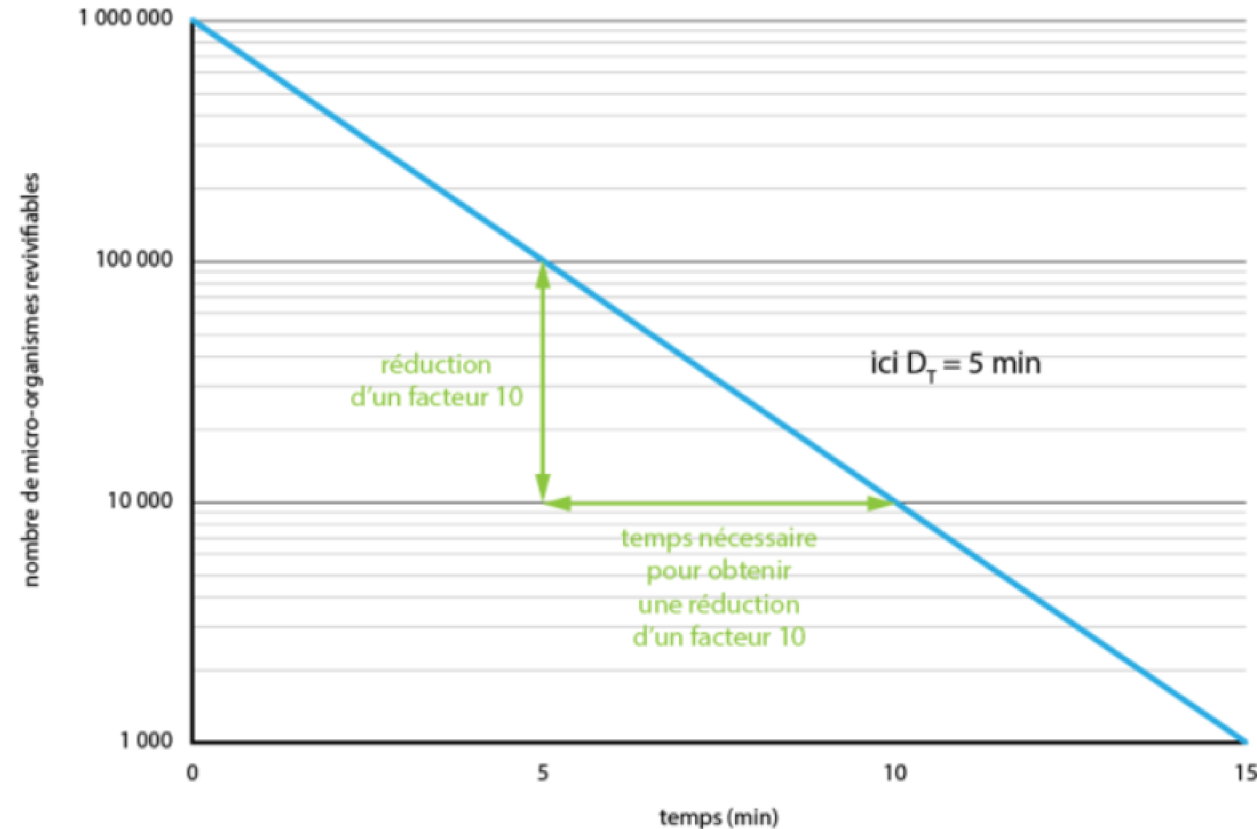


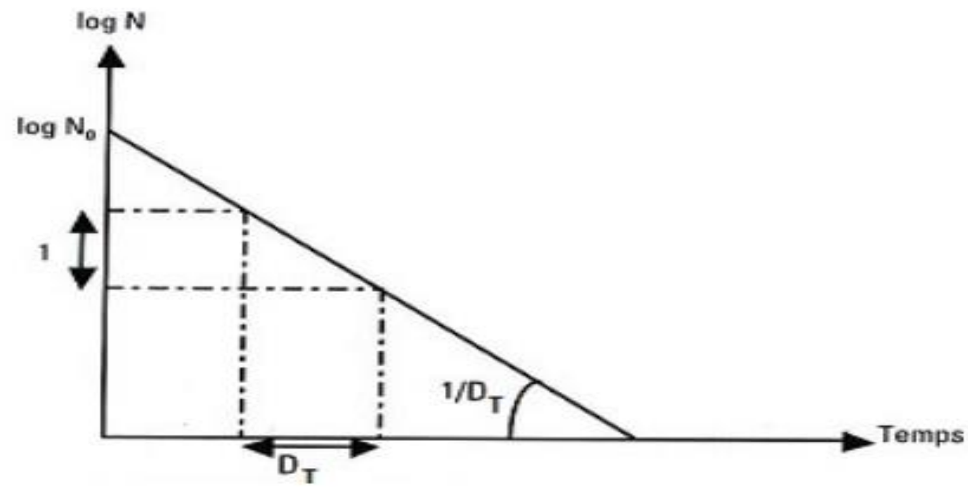
The curves N = f(t) and log N = f(t) are called **survival curves or microbial destruction kinetics**.
The curve log N = f(t) is linear, meaning that microorganisms exposed to a constant lethal temperature follow a **first-order destruction law** as a function of time.

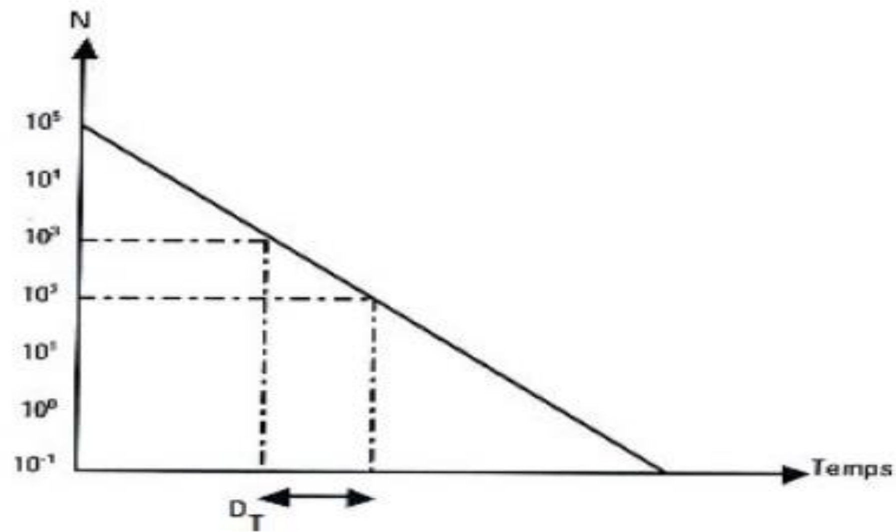
2- Decimal reduction time DT

DT is defined as the heating time at temperature T required to reduce the microbial population by a factor of 10.

- The decimal reduction time at temperature T is the time required to reduce the number of microorganisms by a factor of 10 (by 90%) during treatment at temperature T. This decimal reduction time depends mainly on the type of microorganism, but also on the environment in which it is found (pH, presence of certain ions, fats, Aw,...).







$$\log\left(\frac{N_0}{N}\right) = \frac{t}{D_T} \qquad \frac{N_0}{N} = 10^{\frac{t}{D_T}}$$

- DT provides information on the heat resistance of microorganisms

Exemples : *Clostridium botulinum*	$D_{121,1}$= 6 à 12sec
Clostridium sporogenes (Certains)	$D_{121,1}$> 100sec
Bacillus stearothermophillus	$D_{121,1}$= 250sec

Here, *B. stearothermophilus* is the most heat-resistant bacterium

The equation of the survival curve can therefore ultimately be written as:

$$N = No * 10^{-t/D_T}$$

3- Decimal reduction rate

The decimal reduction rate **n** (or number of decimal reductions), also called pasteurization efficiency E at temperature T, is:

$$n = \log\left(\frac{N_0}{N}\right) \quad \text{ou} \quad E = \log\left(\frac{N_0}{N}\right)$$

$$\frac{N}{N_0} = 10^{-n}$$

The duration of heat treatment at temperature T that allows for the destruction of a proportion of microorganisms equal to n is:

t = n × DT at temperature T

4.Factors affecting heat resistance variation

D, which is a parameter characterizing the heat resistance of microorganisms, depends on several parameters:

•the species of the microorganism in question,
•its physiological state,
•the temperature,
•and the environment in which it is present.

•Thus, DT characterizes the heat resistance of a microorganism under well-defined physicochemical conditions.
Microorganisms are more easily destroyed when they are in the exponential growth phase.

There are two types of flora:

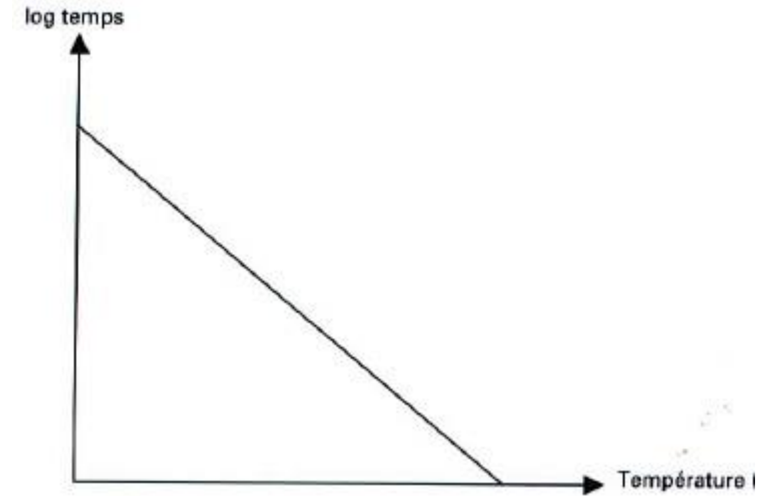
•microorganisms destroyed by treatment at 63°C for 30 minutes (or by equivalent treatment) = heat-sensitive flora;
•microorganisms resistant to treatment at 63°C for 30 minutes (or by equivalent treatment) = heat-resistant flora.

The heat resistance of microorganisms varies according to the physicochemical characteristics of the food, such as pH, water activity (Aw quantifies the availability of water in a food).

- the further the pH of the food is from neutrality, the more sensitive the microorganisms are to heat; this is why foods are classified into three categories according to their pH.
- the lower the Aw of the food, the more heat-resistant the microorganisms are, and therefore the less effective the heat treatment is;
- the higher the fat content of the food, the more resistant the microorganisms will be to heat because lipids are poor conductors of heat.

5.Influence of temperature

5.1. Thermal destruction (or resistance) curve



Study of the influence of heating time (t) on treatment temperature (T) for the same thermal destruction efficiency (n or E = constant)
The figure above shows the relationship between heating time and the lethal exposure temperature required to achieve a given reduction rate (n).
These pairs of heating temperature for a given duration are called heat treatment schedules. These schedules are denoted as (T; t).

Thus, the higher the heating temperature (T), the shorter the thermal destruction time (t). The Bigelow model demonstrates experimentally that, for a given destruction rate (n), the treatment time (t) and temperature T are linked by a linear relationship. In other words, multiple heating schedules can achieve the same thermal destruction efficiency (n = E = constant).
Heating time follows a first-order reduction law as a function of temperature: **log(t) = a.T + b**
This line is called the thermal destruction (or resistance) curve.

The equation of this curve of equivalent schedules is written as:
$(T_1 ; t_1) = (T_2 ; t_2)$

$$t1 = t2 * 10^{(T2 - T1)/z}$$

$$\log (t1/t2) = (T2-T1)/z$$

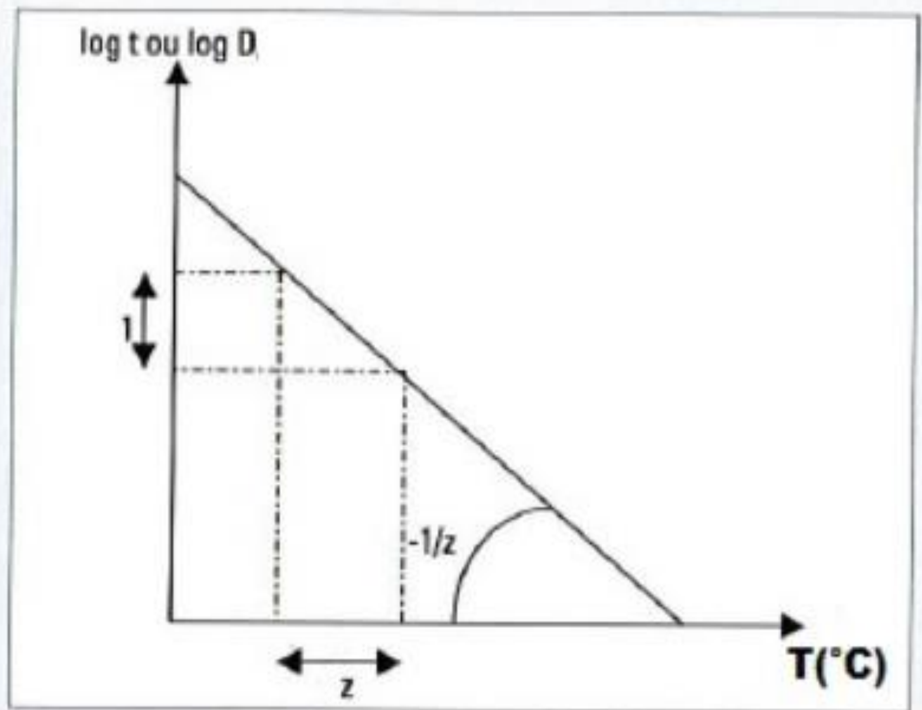
5.2. Thermal factor inactivation z

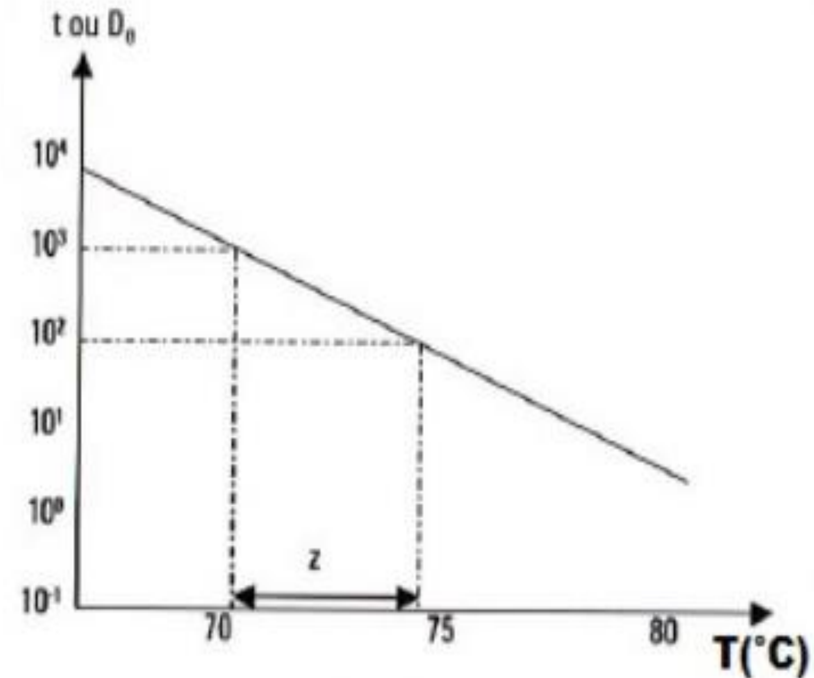
Definition of z:

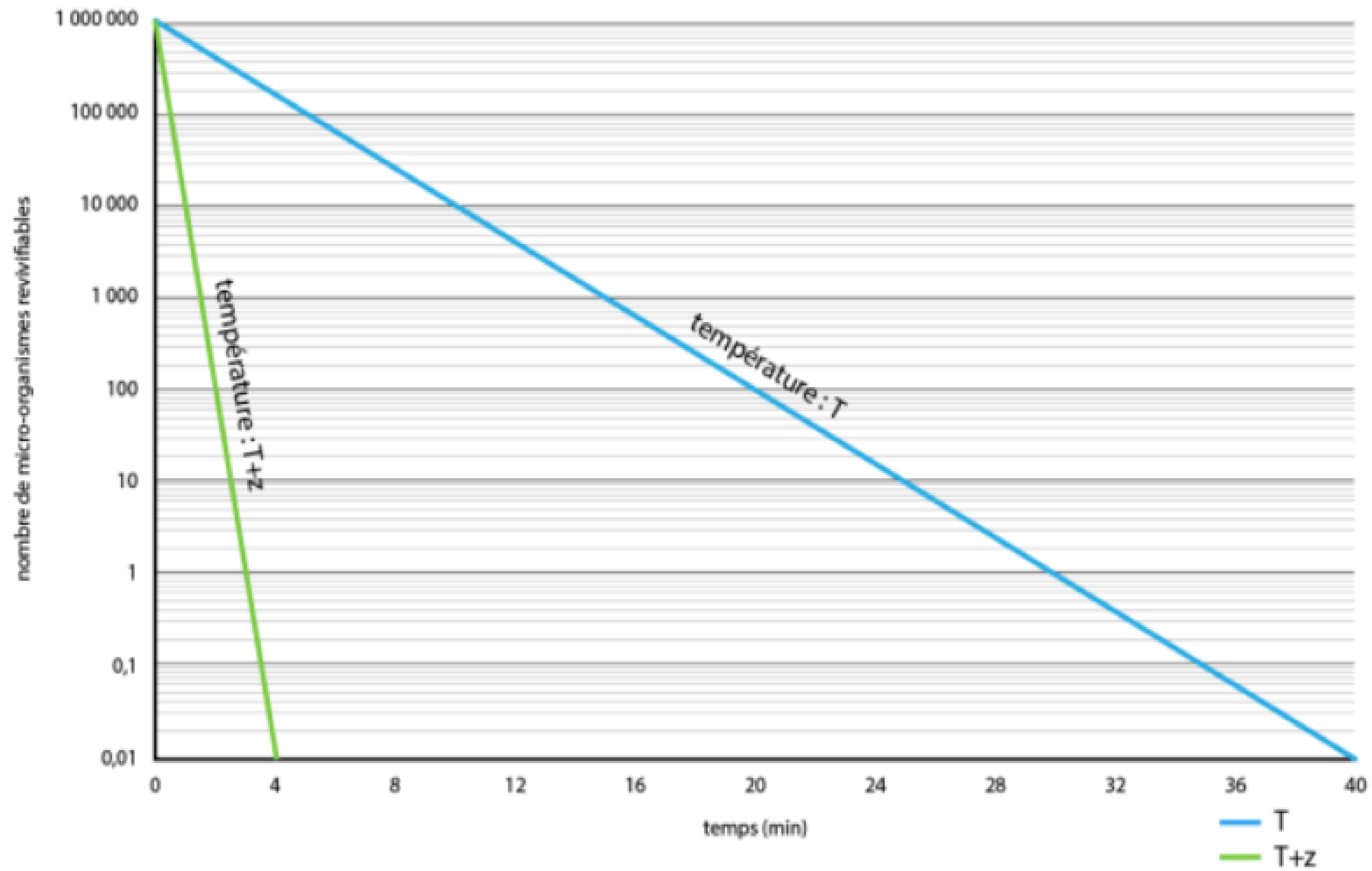
The z-factor is the temperature increase that reduces the heating time t or the decimal reduction time D by a factor of 10, for the same microbial destruction efficacy (n or E = constant).

log(t) =f(T) or log(D) = f(T)

Thus, z is a heat resistance parameter characteristic of each microbial species. In general, z is about 10°C for sporulated forms and 5°C for vegetative forms.







1 000 000
100 000
10 000
1 000
100
10
1
0,1
0,01
nombre de micro-organismes revivifiables
température : T+z
température : T
0
4
8
12
16
20
24
28
32
36
40
temps (min)
T
T+z

PRODUIT	BARÈMES
Jus de fruits	65 °C / 30 min 77 °C / 1 min 88 °C / 15 s
Bière	65-68 °C / 20 min 85-95 °C / 1-2 s
Lait	63 °C / 30 min 71,5 °C / 15 s 85-90 °C / 1-2 s
Œuf liquide	60 °C / 3,5 min 64,4 °C / 2,5 min
Glace	80°C / 15 s

1. Consider a sample contaminated with 10^5 microorganisms.

Determine the heating time and the surviving population after various treatments at $72^{\circ}C$. Given: $D_{72^{\circ}C} = 20\ s$.

Heating time t_{72} (s)	Number of decimal reductions n	Residual surviving population of microorganisms
	0	10^5
	1	
	2	
	3	
	4	
	5	

2. Determine the decimal reduction time (D_T) for each temperature T, given that $D_{60^\circ C} = 600$ seconds and $Z = 5^\circ C$.

T (°C)	D_T in seconds
60	600
65	
70	
75	

We have an initial sample $N_0=10^5$ microorganisms, $D72\circ C=20s$.

A **decimal reduction** means that after a time $t=n\times D_t$, the remaining population is $N=N_0\times10^{-n}$

Here, for each row:

Number of decimal reductions *n is already given*

Heating time $t=n\times D_t$

Surviving population $N=N_0\times10^{-n}$, $D72\circ C=20s$, $N_0=10^5$

t_{72} (s)	n	Surviving population
0	0	10^5
20	1	10^4
40	2	10^3
60	3	10^2
80	4	10^1
100	5	$10^0=1$

Calculating DT at Different Temperatures

The formula for thermal destruction is:

$$\log_{10}\left(\frac{D_T}{D_{T_{\text{ref}}}}\right) = \frac{T_{\text{ref}} - T}{Z}$$

Or more commonly:

$$D_T = D_{T_{\text{ref}}} \times 10^{\frac{T_{\text{ref}} - T}{Z}}$$

Given:

$D_{60^\circ C} = 600\,\text{s}$, $Z = 5^\circ C$, $T_{\text{ref}} = 60$.

- For $T = 65^\circ C$:

$$D_{65} = 600 \times 10^{\frac{60-65}{5}} = 600 \times 10^{-1} = 600 \times 0.1 = 60\,\text{s}$$

- For $T = 70^\circ C$:

$$D_{70} = 600 \times 10^{\frac{60-70}{5}} = 600 \times 10^{-2} = 600 \times 0.01 = 6\,\text{s}$$

- For $T = 75^\circ C$:

$$D_{75} = 600 \times 10^{\frac{60-75}{5}} = 600 \times 10^{-3} = 600 \times 0.001 = 0.6\,\text{s}$$

T (°C)	D_T (s)
60	600
65	60
70	6
75	0.6

Exercise 1

A reduction from 1 to 10^{-9} of an initial population of *Clostridium botulinum* spores is achieved by heating at $120°C$ for 10 seconds.

1- Calculate the decimal reduction time $D_{120°C}$.
2- Calculate the z-value, given that the same reduction is achieved in $27.5\,\mathrm{min}$ at $120°C$?

Exercise 2

A suspension of 10^9 spores/ml of *Clostridium* is heated at $\theta = 116°C$ for 3 minutes. Given: $Z = 8.26°C$ and $D_{120°C} = 11.11\,\mathrm{sec}$.

⇔ Determine the residual population after heating.

Sterilization Value, Pasteurization Value
Definition:
The intensity of a heat treatment, whether for sterilization or pasteurization, is expressed as **a Sterilization Value (F or F0) or a Pasteurization Value (VP)**. It is equal to the duration of a treatment leading to the same reduction in the number of microorganisms (same N/N0) carried out at the reference temperature. It is therefore expressed as equivalent time (minutes or seconds) spent at the reference temperature.

For example, a sterilization value of 3 minutes means that the actual experiment is equivalent to an experiment lasting 3 minutes at a constant temperature equal to the reference temperature (e.g., 121.1°C).

Example:

Clostridium botulinum ($D_{121.1^\circ C} = 0.21\,\text{min}, z = 10^\circ C$): we want to reduce from 10^{12} spores/g to 1 spore/g:

At the reference temperature ($T = T_{\text{ref}} = 121.1^\circ C$), the time required is:

$$t = n \times D_T = (12 - 0) \times 0.21\,\text{min} = 12 \times 0.21 = 2.52\,\text{min}$$

At 131.1°C, $T = T_{\text{ref}} + z$, the time required to achieve the same reduction is:

$$t = n \times D_{T_{\text{ref}}+z} = 12 \times \frac{D_{T_{\text{ref}}}}{10} = 12 \times 0.021 = 0.252\,\text{min}$$

These two treatments are equivalent; they result in the same reduction in the number of microorganisms, so they have the same sterilization value:

$$F_0 = t \times 10^{\frac{T - T_{\text{ref}}}{z}} = 2.52\,\text{min}$$

Damage Caused by Heat Treatments

◆ **Degradation of ribosomes and ribosomal RNA**

Numerous studies have shown that heat causes the degradation of ribosomes and ribosomal RNA. This alteration primarily affects the small subunit of the ribosome (30S fraction of the ribosome and 16S fraction of ribosomal RNA), which can be completely degraded, whereas the large subunit is only partially affected.

◆ **Alterations of cytoplasmic membranes**

Heat treatment leads to functional alterations of the bacterial membrane. *Staphylococcus aureus* loses 30% of its membrane lipids during heating.

◆ **Metabolic damage**

This is mainly due to the denaturation of heat-labile enzymes: aldolase and lactate dehydrogenase, NADH oxidase and cytochrome c reductase, the enzyme involved in pantothenate synthesis, catalase, superoxide dismutase, etc.

◆ **DNA degradation**

Heat can theoretically cause DNA strand breakage through the activation of nucleases, but it appears that this molecule remains relatively stable *in vivo*.

Reference temperature and reference microorganism

In pasteurization, the reference temperature T_{ref} is:

- $T_{\text{ref}} = 70°C$, particularly when the product is contaminated by bacteria.
- $T_{\text{ref}} = 60°C$ or $65°C$ for acidic products colonized by yeasts or molds.

The z-factor also depends on the microorganisms colonizing the product:

- $z = 10°C$ in general (bacteria)
- $z = 5$ to $7°C$ in general for acidic products colonized by yeasts and molds

Reference Germ:

This is the most heat-resistant pathogenic microorganism that colonizes the product to be pasteurized; its choice therefore depends on the product.

Example:

- For the pasteurization of **meat products and ready meals, Streptococcus (or Enterococcus) faecalis** is often chosen.
- For dairy products: *Mycobacterium tuberculosis* (tubercle bacillus); *E. coli* may also be chosen.

The most important factors determining microbial resistance to heat

Introduction

Heat resistance (also known as thermotolerance) is the ability of microorganisms to survive exposure to high temperatures that would normally be lethal. Understanding the factors that determine this resistance is crucial for food safety, industrial biotechnology, sterilization processes, and clinical settings. Unlike resistance to antimicrobial drugs, which is often mediated by specific acquired genes, resistance to heat is a complex, multifactorial trait involving structural, molecular, and environmental factors

1. Sporulation: the ultimate structural resistance

The most significant factor differentiating heat resistance among bacteria is the ability to form **endospores**.

•**What are spores?** Bacterial genera such as *bacillus*, *geobacillus*, and *clostridium* can form dormant endospores when nutrients are scarce. These structures are designed for survival, not reproduction, and are arguably the most heat-resistant life forms known .

•**The spore structure**: the core of a spore is dehydrated and contains specific molecules that protect DNA. It is surrounded by a thick cortex and a proteinaceous coat, which act as barriers.

•**Variability in spore resistance**: not all spores are equal. The intrinsic heat resistance of spores varies significantly between **species and even strains**. For example, spores of *geobacillus stearothermophilus* are notoriously heat-resistant and are used as biological indicators to validate autoclave function, while others are more sensitive .

•**Sporulation conditions**: the temperature at which sporulation occurs directly impacts the resulting spore's resistance. Research shows a direct relationship between sporulation temperature and the subsequent spore-killing temperature; spores formed at higher temperatures tend to be more heat-resistant . exposure to other stressors during formation, such as ethanol, can perturb membrane development and result in more temperature-sensitive spores

2. Molecular and cellular mechanisms

When vegetative (active) cells or spores are subjected to heat, their survival depends on a cascade of molecular responses.

2.1. Heat Shock Proteins (HSPs) and Chaperones

The immediate response to a temperature increase is the production of **Heat Shock Proteins**.

•**Function**: HSPs, such as the **GroES/GroEL** system and **GrpE**, act as molecular chaperones. They help other proteins maintain their correct three-dimensional structure (folding) and prevent them from aggregating when heat tries to denature them .

•**Evidence**: Studies on thermoadapted bacteria show that overexpression of genes for chaperone systems (like GroES-GrpE) is a primary mechanism for boosting survival at extreme temperatures (e.g., up to 85°C) . The greater the capacity to repair protein damage, the higher the heat resistance .

2.2. Membrane stabilization and composition

The cell membrane is a primary site of heat damage. High temperatures increase membrane fluidity, which can lead to loss of function and cell death.

•**Lipid Modification**: Bacteria adapt by altering the composition of their membrane lipids. For instance, they may increase the saturation of fatty acids to maintain proper membrane stability at higher temperatures.

•**Specific Enzymes**: Key enzymes play a role here. In *Zymomonas mobilis*, a decrease in the activity of **diacylglycerol kinase** (encoded by *dgkA*) was found to alter lipopolysaccharide (LPS) structure, likely strengthening the membrane

2.3. DNA protection and repair

Heat can cause DNA damage (denaturation and breaks).

•**Protection in spores**: in bacterial spores, dna is saturated with small, acid-soluble proteins (sasps) that physically alter the dna's conformation and protect it from heat damage.

•**Repair systems**: upon germination, spores rely on robust **dna repair systems** to fix the damage that occurred during the dormant state . vegetative cells also upregulate dna repair enzymes under heat stress.

2.4. Compatible solutes and osmolytes

To cope with the stress, some bacteria accumulate small molecules called compatible solutes.

•**Function**: molecules like **trehalose** stabilize proteins and membranes during heat stress, preventing denaturation . this mechanism is also used to combat osmotic and desiccation stress, providing cross-resistance.

3. Evolutionary and genetic factors
A microbe's ability to resist heat is not static; it can change through evolution and genetic adaptation.

3.1. Adaptive laboratory evolution (ALE)
When bacteria are exposed to gradually increasing temperatures over many generations, they can adapt.
•**Genomic changes**: studies on *geobacillus stearothermophilus* show that prolonged heat adaptation (from 70°c to 80°c) leads to specific genomic changes, such as the deletion of transposable elements (which reduces genomic instability) and mutations that enhance metabolism and stress response .
•**Trade-offs**: thermal adaptation can come with trade-offs. For example, a cold-tolerant bacterium (*B. Mycoides*) that adapts to higher temperatures may see a decrease in the range of temperatures it can grow at (reduced "operational niche width") .

3.2. Genomic islands and core genes
Thermotolerance is often encoded in the genome.
•**Thermotolerance genomic islands**: some bacteria possess clusters of genes specifically dedicated to heat resistance that can be acquired horizontally .
•**Convergent evolution**: interestingly, different species and independently evolved mutants often arrive at similar solutions for heat resistance. Genes involved in **membrane stabilization, protein quality control, and transporters** are consistently found to be key, suggesting that the molecular diversity of thermal adaptation strategies is limited

4. Environmental and physiological conditions
The state of the microbe and its environment at the time of heat exposure critically determine the outcome.

4.1. Growth phase and prior stress

- **Stationary phase**: cells in the stationary phase (dormant, not dividing) are generally more heat-resistant than cells in the exponential growth phase (actively dividing).
- **Heat shock pretreatment**: exposing cells to a mild, sub-lethal heat shock before a severe challenge can induce the production of hsps and significantly increase survival rates. This acquired thermotolerance was observed in *bacillus subtilis* during sporulation, where a 30-minute pretreatment at 48°C led to the overexpression of 60 proteins and enhanced spore resistance .
- **Cross-resistance**: prior exposure to other stresses (like acid or osmotic stress) can sometimes activate pathways that also protect against heat, a phenomenon known as cross-resistance .

4.2. Food matrix and physicochemical factors
In food and natural environments, the surrounding matrix plays a huge role in heat resistance .

- **Water activity (aw)**: lower water activity (drier environments) dramatically increases heat resistance. This is why pathogens in low-moisture foods like chocolate or powdered infant formula are much harder to kill with heat.
- **Fat content**: high fat content can protect bacteria by reducing the water activity in their immediate microenvironment.
- **Ph**: the acidity or alkalinity of the medium can synergize with heat; heat is often more lethal at extreme ph values.

Thermal Death Point (TDP)

•**Definition:** The Thermal Death Point (TDP) is the **lowest** temperature required to kill all microorganisms in a liquid suspension within a specific time frame—exactly **10 minutes**.

•**The "Breaking Point":** It represents the specific temperature threshold at which a microorganism cannot survive, acting as its "breaking point" under heat stress.

•**Standardized measurement:** Unlike variable pasteurization processes, TDP is a standardized reference point with a fixed time (10 minutes), allowing for direct comparison of the heat resistance of different microbes.

•**Complete sterilization:** The goal of reaching the TDP is **complete elimination** of all microorganisms, including bacteria, yeasts, molds, and the more heat-resistant spore-forming bacteria.

Factors affecting thermal death point

- **pH level (Acidity):** Microorganisms in **acidic environments** (low pH) are generally more sensitive to heat. This lowers the TDP, meaning acidic foods require less heat to be sterilized.

- **Water activity:** High water activity (more available water) makes microbes more heat-sensitive, as water helps transfer heat into the cell. **Low-moisture environments** can protect microbes, raising their TDP.

- **Type and concentration of microbes:** Different microbes have different resistances. **Spore-forming bacteria** (e.g., *Clostridium botulinum*) have a much higher TDP than vegetative cells, making them the target for processes like commercial canning.

Practical applications in food processing

- **Commercial Canning:** TDP data is used to establish the minimum processing temperatures required to destroy the most heat-resistant pathogens (like *C. botulinum* spores), especially in low-acid foods.

- **Pasteurization:** While pasteurization uses varying time-temperature combinations, its parameters are based on the TDP principles of target pathogens to eliminate harmful bacteria while preserving food quality (e.g., in milk).

- **Juice Processing:** TDP values help manufacturers determine treatments that eliminate pathogens (like *E. coli* and *Salmonella*) without compromising the fresh taste and nutritional value of the juice.

Laboratory determination of TDP

•**Standardized Suspensions:** Scientists prepare samples with a known concentration of the target microorganism.

•**Controlled Exposure:** These samples are exposed to a range of specific temperatures for exactly **10 minutes**.

•**Determining Survivability:** After heating, the samples are cultured in growth media and incubated. The TDP is the lowest temperature at which no viable microorganisms grow.

Laboratory Determination of TDP

TDP = Lowest Temperature with No Microbial Growth

Industrial significance and quality control

- **Foundation for critical limits:** TDP data is essential for establishing Critical Control Points (CCPs) in HACCP plans, specifically defining the minimum temperature requirements for heat treatment equipment.

- **Regulatory standards:** Agencies like the FDA use TDP data to create science-based food safety regulations and guidelines for processing.

- **Economic optimization:** Understanding TDP allows processors to use the minimum heat necessary for safety, avoiding over-processing that wastes energy and degrades product quality, thus balancing safety with efficiency.

Microbial inactivation kinetics and survival curve profiles

1. Introduction to inactivation kinetics

When a microbial population is subjected to a lethal agent (heat, UV radiation, chemical disinfectant, etc.), its destruction is not instantaneous. Inactivation kinetics is the study of the rate at which microorganisms lose their ability to reproduce (referred to as "death" or "inactivation") over time.

The fundamental tool for visualizing these kinetics is the survival curve. This is the graphical representation of the number of survivors as a function of exposure time to the stressing agent. Due to the very large microbial populations, the number of survivors is almost always plotted on a logarithmic scale ($\log_{10}$), while time is plotted on an arithmetic scale. This is known as a semi-logarithmic representation.

2. The historical model: linear logarithmic kinetics (First-Order)

Historically, and still very often in the industry today (notably for canning), it is considered that the destruction of microorganisms follows so-called **first-order kinetics**.

2.1. Concept

In this model, it is assumed that all microorganisms in a population have the same sensitivity to the lethal agent and that the probability of a cell being inactivated is constant over time. Therefore, the destruction rate is proportional to the number of remaining survivors.

2.2. Graphical representation

On a semi-log graph, this kinetics translates into a straight line. This is the simplest and best-known form of survival curves.

2.3. The key parameter: The D-value

The linearity of the curve allows the definition of a universal parameter: the decimal reduction time, or D-value.

- Definition: It is the time required, at a given temperature (or agent concentration), to reduce the microbial population by 90%, i.e., by a factor of 10 (in other words, a 1-log_{10} reduction).

- Usefulness: The higher the D-value, the more resistant the microorganism is to the agent under the given conditions. For example, one might refer to `$D_{121}$°C` for moist heat, which is the time at 121°C to reduce a population of a given strain.

$$\log_{10} N(t) = \log_{10} N_0 - D_t$$

Or alternatively:

$$N(t) = N_0 \cdot 10^{-Dt}$$

where N(t) is the population at time t, and N_0 is the initial population

This model is still widely used because it is simple and effective.

3. Profiles of non-linear survival curves

However, as soon as we closely observe inactivation kinetics, especially for mild treatments or complex populations, we realize that reality is more nuanced. Curves are not always straight lines. We then observe deviations, the most common being the shoulder and the tail.

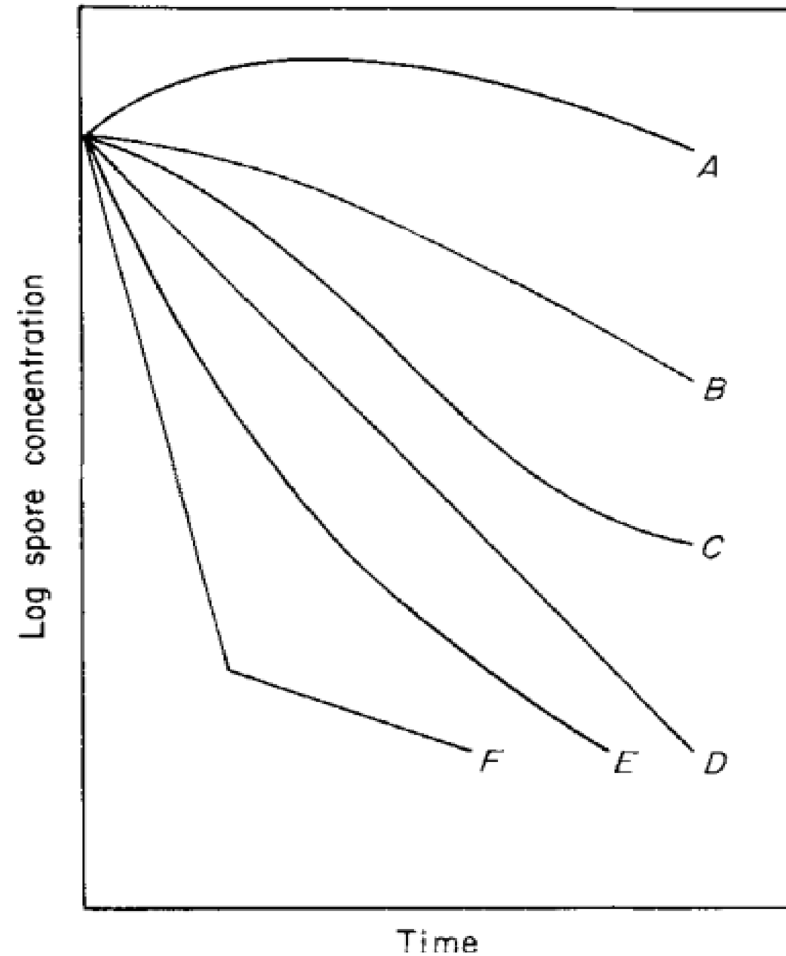


Fig. 1. Types of survival curves. *A* and *B*, 'shoulder'; *C*, 'sigmoid'; *D*, logarithmic curve; *E*, 'upward concavity'; *F*, 'biphasic curve' with a 'tail'.

3.1. Curves with a Shoulder

The curve shows an initial phase where the microbial population decreases very slowly, or not at all (lag phase or "shoulder"), before exponential (linear) destruction begins.

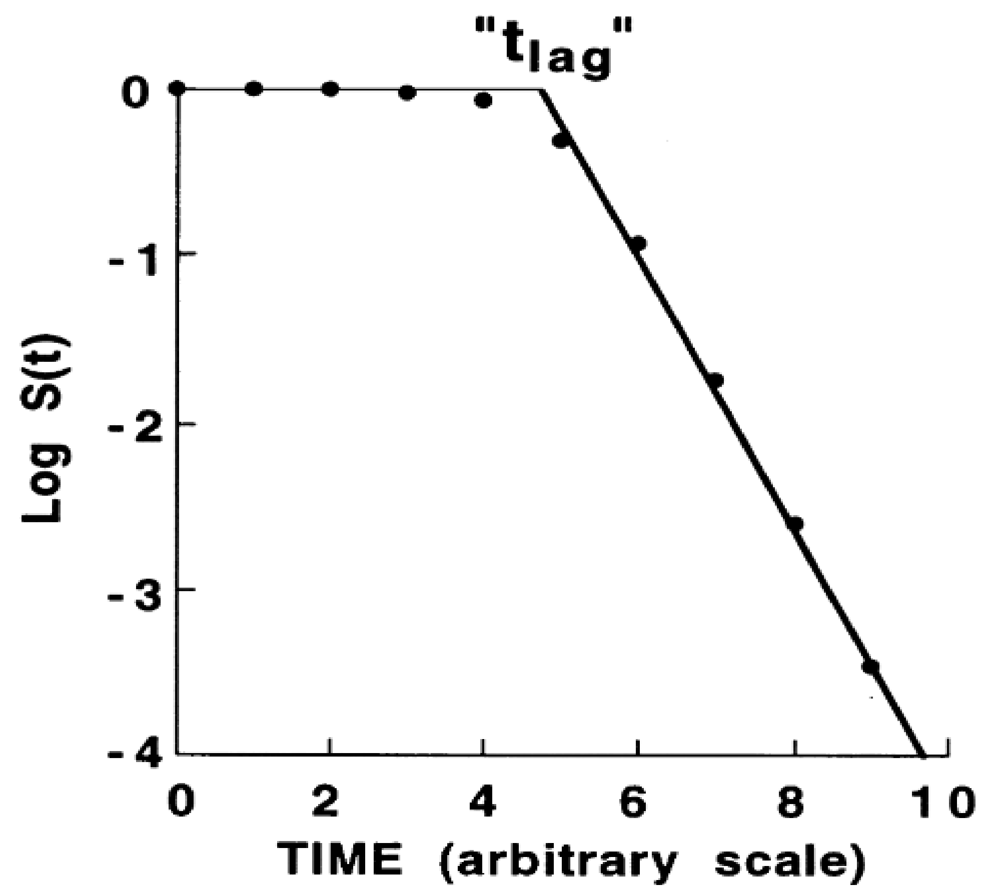
- Interpretation:

- Repair: Cells activate their damage repair systems (e.g., DNA repair after an insult). As long as the systems keep up, the cell survives.

- Accumulation: Death only occurs after the accumulation of a certain number of sublethal damages within the cell.

- Aggregation: In the case of spores or aggregated cells, the agent must first "dissociate" or act on the outer layers before reaching all cells.

- Agents involved: Common in thermal bacteriology for moderate temperatures, or with certain chemical agents .


"t_{lag}"
0
-1
-2
-3
-4
Log S(t)
0
2
4
6
8
10
TIME (arbitrary scale)

3.2. Curves with tailing

The curve initially descends linearly, but then the slope flattens out significantly, forming a "tail" or a "plateau" where a small subpopulation seems to become highly resistant .

- Interpretation:

- Phenotypic heterogeneity: Within a genetically identical population, some individuals may enter a different physiological state (e.g., stationary phase, spore formation) that makes them more robust.

- Genetic heterogeneity: The population is a mixture of strains or species with different resistances. The most resistant ones eventually become dominant among the survivors .

- Physical protection: Cells may be located in microscopic environments that protect them (biofilms, organic matter, particles).

- Agents involved: Very frequent for UV treatments, chemical disinfectants, and heat in a protective environment .

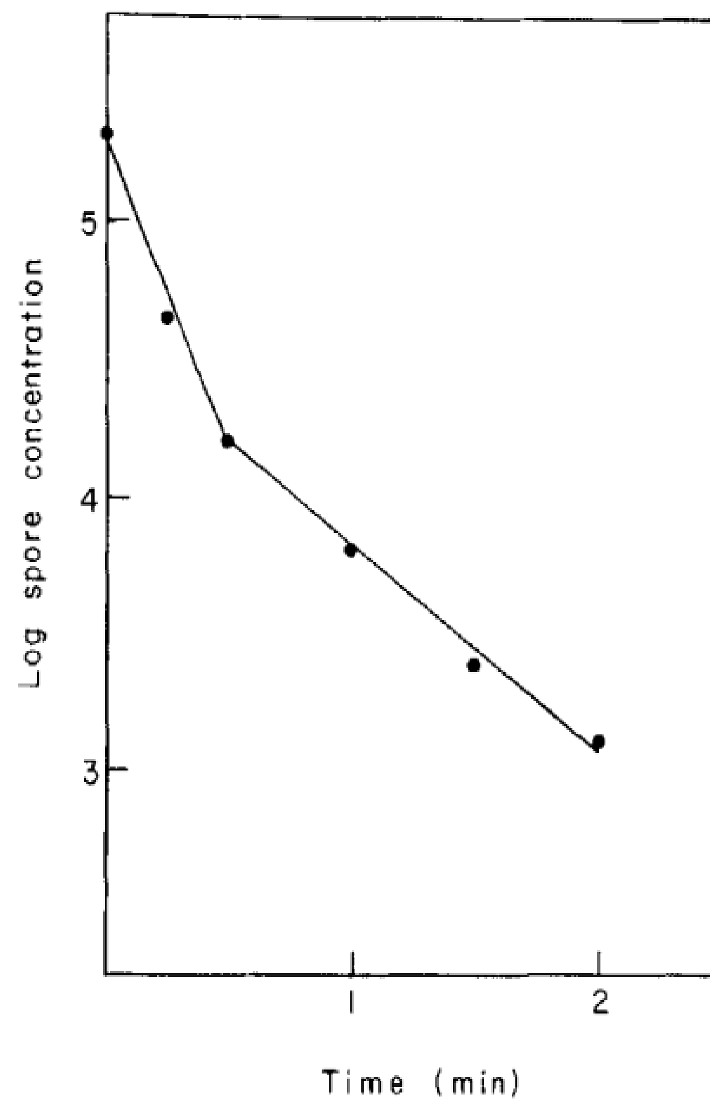


Fig. 3. Survival curve of a suspension prepared by mixing suspensions of rough variants, transparent and opaque, of *Bacillus stearothermophilus* NCA 1518 spores treated with sodium hypochlorite (1000 mg/l free chlorine, pH 7·0, in 0·5 mol/l phosphate buffer, 25 °C).

- Many scientists consider the tailing phenomenon (the tail of the survival curve) to be an artefact, rather than a true biological characteristic.

- One of the main causes of this artefact is genetic heterogeneity within the bacterial population subjected to the treatment.

- Mixing two populations with different levels of resistance produces a biphasic survival curve.

- The height of the tail (its extrapolation back to time zero) remains constant even when the treatment intensity is modified; it corresponds to the initial concentration of the most resistant subpopulation.

- Mixing several populations (multiphasic) can produce curves that are difficult to distinguish from the "concave upward" curves observed in other models.

- A concrete example is the variation within a single strain of *Bacillus stearothermophilus* (resistant R and sensitive S variants), the mixing of which artificially reproduces these tailing curves (figure) .

3.3. Sigmoid and complex curves

It is also possible to observe curves combining both effects: an initial shoulder, followed by a phase of rapid destruction, and then a tail. These curves are called "sigmoid". One can also observe curves with successive slope changes, indicating subpopulations with varying resistance levels .

4. Interpretation and modern modeling

4.1. Biological reality: heterogeneity

Modern science recognizes that the shape of the survival curve is a direct reflection of the distribution of individual resistances within the microbial population . A population is not an assembly of identical clones, but a collection of individuals with natural variability in their sensitivity.

- A straight line suggests that the probability of death is constant: resistance is homogeneous.

- A shoulder suggests that death requires the accumulation of a minimum number of "hits" (quanta of damage) .

- A tail suggests an "asymmetric" distribution of resistance, with a tail of the population being extremely robust .

4.2. The Weibull model

Faced with these non-linearities, linear models (based on the D-value) are insufficient. The model most commonly used to describe these complex curves is **the Weibull model**.

It is a flexible equation that can take different shapes (linear, concave, convex) through two parameters:

$$\log_{10} N(t) = \log_{10} N_0 - (\delta/t)^p$$

- δ (delta) : is a scale parameter (related to overall resistance, similar to the concept of D).

- p (shape) : is a shape parameter.

  - If $p = 1$, the curve is a straight line (classic linear model).

  - If $p < 1$, the curve is concave (upward curvature): it exhibits tailing. This indicates that the most resistant cells become increasingly predominant over time .

  - If $p > 1$, the curve is convex (downward curvature): it exhibits a shoulder. This indicates that the accumulation of damage is necessary before death accelerates .

Inactivation Agent	Frequent Curve Profiles	Common Interpretation
Heat (mild treatment)	Shoulder + linear phase, or Weibull $p>1$	Repair of thermal damage, heat shock proteins .
Heat (intense treatment)	Linear ($p=1$)	Massive and rapid destruction, individual variability is masked .
UV Radiation	Pronounced tailing ($p<1$)	Presence of highly efficient DNA repair mechanisms in a small fraction of the population (survival "plateau") .
Plasma / UV	Can be complex (sigmoid)	Combination of mechanisms (UV action + reactive oxygen species) .
Chemical Agents	Often with shoulder or tailing	Agent diffusion, saturation of binding sites, metabolization of the product, or persister phenotypes .

4. Interpreting deviations from linearity: methodological artifacts

While biological interpretations (population heterogeneity, accumulation of damage) explain a large part of the non-linearities, it is essential, as a scientist, to consider another possibility: Is the observed curve a faithful reflection of biological reality, or is it distorted by the way we conducted the experiment?

Before concluding a complex biological phenomenon (such as a distribution of resistances following a Weibull model), the experimenter must systematically rule out the possibility of a methodological artifact. Foundational scientific literature emphasizes that while it is *unlikely* that all observations of non-linearity are due to artifacts, this verification remains a key step in any rigorous analysis.

❖ Definition

A methodological artifact, in the context of survival curves, is a distortion of the curve that is not due to the actual behavior of the microorganism, but is caused by a flaw or limitation in the experimental method used.

In other words, it's a "false friend": the curve tells us something (for example, that the bacteria suddenly become very resistant), when in reality, it is our way of measuring or treating the sample that creates this illusion.

4.1. Artifacts related to experimental conditions

These artifacts occur when the medium or treatment conditions are not perfectly controlled.

- **Evolution of temperature or agent concentration:**

To be valid, an inactivation kinetic study assumes that the intensity of the lethal agent (temperature, disinfectant concentration, UV dose) is constant and uniform throughout the treatment duration.

Example: If you are studying thermal inactivation and you introduce a tube containing your sample into a water bath, the contents of the tube do not instantly go from 20°C to 60°C. There is a temperature come-up time. During this phase, the microorganism is exposed to increasing sublethal temperatures. On the survival curve, this will manifest as an apparent shoulder, which is not due to the biological resistance of the cells, but to a purely physical delay in the application of the stress.

For chemical agents: If the diffusion of the disinfectant into the sample is not instantaneous, the effective concentration in contact with the cells increases gradually, also creating an artificial shoulder.

- **Protection by the medium**

The composition of the sample can interact with the lethal agent.

Example: For a UV or plasma treatment, the presence of solid particles or organic matter can "shade" the microorganisms that are behind them. For a chemical disinfectant, organic matter can neutralize part of the product before it reaches the cells. This can create an artificial **tail**, because a subpopulation appears protected when it is simply physically masked or chemically "buffered" by its environment.

4.2. Artifacts related to enumeration techniques

The way we measure survivors is a major source of errors and bias.

- **Detection limit and "floor" effect:**

Petri dish counting techniques have a detection limit. You cannot count less than one colony. If your initial population is 10^6 CFU/mL and your technique cannot detect below 10 CFU/mL, you cannot follow inactivation beyond a 5-log reduction.

If the curve seems to show a tail that reaches a plateau at the detection limit, it is impossible to know whether this plateau is real (a highly resistant subpopulation) or if it is simply an artifact because you can no longer measure values below that point. The observed "plateau" is then merely the "noise floor" of your measurement method.

- **Cellular resuscitation and repair:**

Certain treatments (notably UV or some disinfectants) damage cells without killing them instantly. These cells are termed "stressed" or "viable but non-culturable" (VBNC). On a standard culture medium, they will not form a colony and will therefore be counted as "dead."
If the culture medium used is not sufficiently rich or adapted to allow the resuscitation of these stressed cells, the number of survivors will be underestimated. Conversely, if the test or post-incubation conditions allow for repair, survival may be overestimated. This can artificially alter the slope of the curve, particularly in its final part (the tail).

- **Cell Aggregation:**

If cells tend to form clumps or chains, a single colony on a Petri dish may originate from a clump of several cells, not just one. This leads to an underestimation of the total number of viable cells.
During treatment, if the lethal agent kills the cells on the surface of the clump but those in the center survive, counting becomes very erratic and may suggest an artificial tail. This happens because each colony formed still represents several surviving cells.