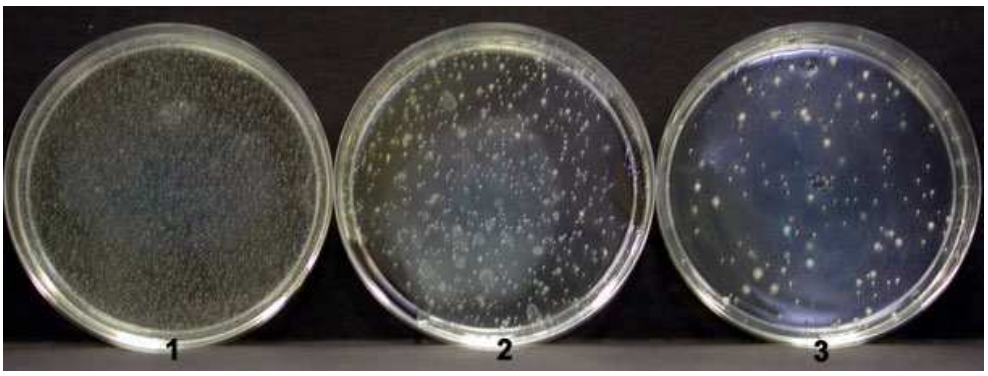
HOW TO SOLVE PRACTICAL ASPECTS OF MICROBIOLOGY

2. BASIC METHODS FOR MICROBIAL ENUMERATION



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2. BASIC METHODS FOR MICROBIAL ENUMERATION

There are a number of different methods to enumerate the microorganisms that are present in a given population. Each method has its own peculiarities to transform the data obtained (colony forming units, total microorganisms, etc...) in microbial density of the sample.

A simple method for the enumeration of bacteria and fungi is based on the **quantification of colony-forming units (CFU) per ml or g of sample**. For this, we must prepare serial dilutions of the sample, plate the diluted suspensions and count the number of colony forming units. Figure 1 shows a scheme of the method.

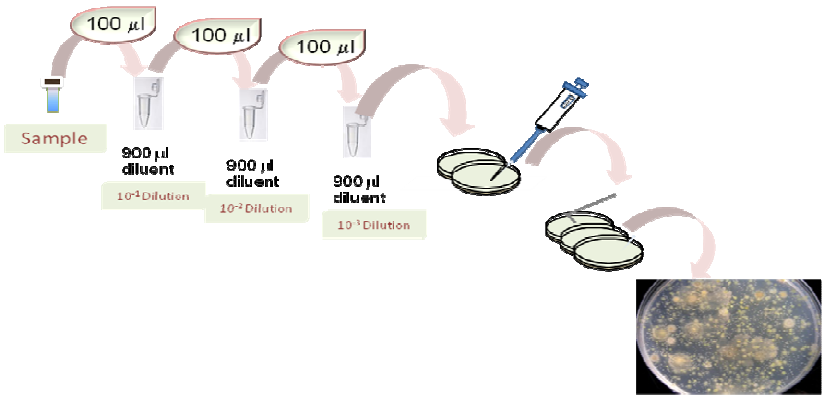


Figure 1. CFU count method scheme.

In the previous chapter, we have solved some problems based on the use of this method. However, it should be noted that, normally, 3 or 5 plates per dilution are used.

If we perform different decimal dilutions and inoculate more than one plate per dilution, we have different possibilities to interpret the results. It is important that the number of colonies developing on the plates not be too large or too small. The usual practice, which is the most valid statistically, is to count colonies only on plates that have between 30 and 300 colonies. For determining the number of bacteria per mL in the original solution, we have to take into account the average colonies of the selected dilution, the dilution factor and the volume plated.

$$\text{CFU/ml} = \frac{\textbf{A} \text{ colonies (average)}}{\textbf{B} \text{ volume plated (ml)}} \text{ X } \textbf{DF} \text{ (Dilution factor)}$$

$$\text{CFU/ml} = \frac{\textbf{A} \text{ ecolonies (average)}}{\textbf{B} \text{ volume plated (ml)}} \text{X} \frac{1}{\textbf{CF} \text{ (Concentration factor)}}$$

With the insights acquired in the previous chapter, you should resolve the following problem:

2.1. Calculate the bacterial density, expressed as CFU/ml, for the three different samples presented in the table. The dilutions used and the number of CFU obtained in each case are also presented in the table. In all cases, the spread volume was 100 µl/plate.

Sample	Dilution	Plate 1	Plate 2	Plate 3
1	10^{-3}	1816	1698	1885
	10^{-4}	180	159	186
	10^{-5}	16	19	10
2	10^{-2}	475	477	480
	10^{-3}	45	48	51
	10^{-4}	5	10	4
3	10^{0}	335	328	324
	10^{-1}	32	28	29
	10^{-2}	5	3	2

An interesting question arises when microbial density is below the detection limit, the lowest number of CFU that can be detected by the method used. How must be the results expressed in this situation? Try to solve the following problem:

2.2. Which is the detection limit of the method if we inoculate 3 plates of a sample with 100 µl per plate?

A variant of the standard CFU method is the quantification in microdrops. The main differences are that, in this case, the plated volume is lower (usually, 10 or 20 µl) and it is not extended in the plate. The method is schematically described in Figure 2.

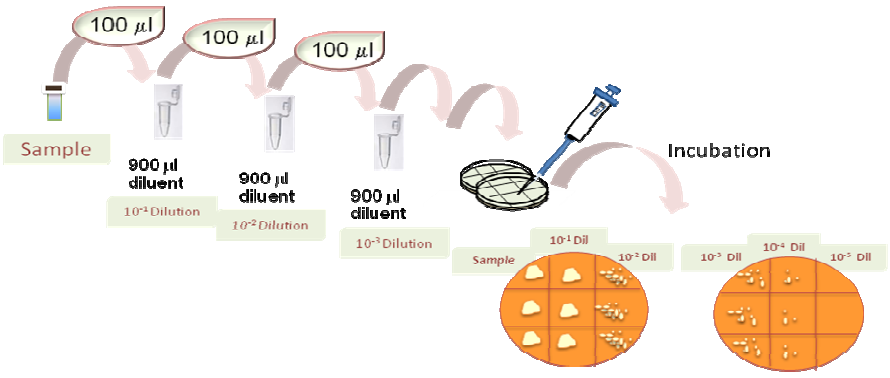


Figure2. Scheme of the microdrop method for CFU count.

2.3. The microdrop method was used to determine the bacterial density of a suspension. The dilutions employed and the results are shown in the table. The plated volume was 10 µl.

Dilution	Count 1	Count 2	Count 3
10^{-3}	180	159	186
10^{-4}	16	19	10

What is the bacterial density of the sample?

Another usual technique to enumerate microbes is the **Most Probable Number (MPN) method**. This is an enumeration method based on the statistic, and it allows estimating the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in serial dilutions. First, decimal dilutions of the sample are prepared and then 1 ml of each dilution is inoculated into 3 broth culture tubes (different numbers of replicates and dilution series can be used). After incubation, tubes are examined for turbidity and those that are positive are recorded for each dilution. For example, if all tubes show growth, the results will be noted as 333. Once the results have been noted, the MPN table (MacGrady table) should be used to determine the most probable number of microorganisms for ml or g.

Solve the following problems using the MacGrady table attached below.

2.4. The 1:10 and 1:100 dilutions have been prepared from a water sample. Moreover, 3 sets of 3 tubes were prepared, each containing 10 ml of nutrient broth. The first set of tubes was directly inoculated with the water sample; the second set was inoculated with the 1:10 dilution and the third, with the 1:100 dilution. The inoculated volume was 1 ml in all cases. The tubes were incubated for 48 h at 20°C and, after this period, each tube was examined for turbidity (growth). From the results shown in the table, what is the bacterial density of the water sample?

Procedure	**Results***		
Sample	G	G	G
1:10 Dilution	G	G	G
1:100 Dilution	NG	G	NG

*G = growth = turbidity; NG = not growth = transparent

2.5. Using the example given above, if growth was not detected in any tube, which would be the bacterial density?

MacGrady Table (1 ml/tube, 3 tubes/dilution, 3 ten-fold dilutions). **Results expressed as bacteria/ml**

Number of positive tubes	MPN	Number of positive tubes	NMP	Number of positive tubes	NMP
000	-	**201**	1.4	**302**	6.5
001	0.3	**202**	2.0	**310**	4.5
010	0.3	**210**	1.5	**311**	7.5
011	0.6	**211**	2.0	**312**	11.5
020	0.6	**212**	3.0	**313**	16.0
100	0.4	**220**	2.0	**320**	9.5
101	0.7	**221**	3.0	**321**	15.0
102	1.1	**222**	3.5	**322**	20.0
110	0.7	**223**	4.0	**323**	30.0
111	1.1	**230**	3.0	**330**	25.0
120	1.1	**231**	3.5	**331**	45.0
121	1.5	**232**	4.0	**332**	110.0
130	1.6	**300**	2.5	**333**	>140.0
200	0.9	**301**	4.0		

Other alternatives to determine the total number of microorganisms in a sample are the **enumeration methods based in microscopy** (photonics, epifluorescence, ...).

The relatively large organisms such as yeast or protists can be enumerated using counting chambers (or haemocytometer) (Figure 3). In this procedure, the number of cells in a given volume of liquid culture is counted directly in various microscope fields. The main disadvantage of this method is that usually it is not possible to distinguish between live and dead cells.

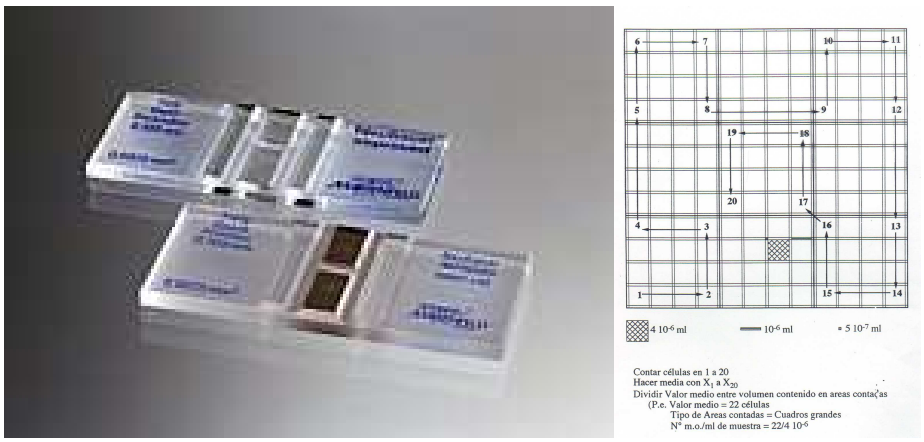


Figure 3. Some cells counting chambers and scheme to select grids.

The different types of counting chambers differ in the counting grids and in the depth of the chambers. The volume of each grid must be known, in order to determine the microbial concentration. Moreover, prior to the enumeration the sample can be diluted or concentrated, so the equations to be used are:

$$\text{Microorganisms/ml} = \frac{\text{Average number of microorganisms/grid}}{\text{Grid volume (ml)}} \times \text{Dilution factor}$$

$$\text{Microorganisms/ml} = \frac{\text{Average number of microorganisms/grid}}{\text{Grid volume (ml)}} \times \frac{1}{\text{Concentration factor}}$$

Attention should be given to the units of each factor in the equation. The final result is expressed as number of organisms per volume of sample.

2.6. To determine the yeast concentration in a dense suspension, this suspension was diluted 1,000 times before preparing the counting chamber. Then, 20 grids were counted and the results were recorded in the table that appears below. If the chamber factor given by the manufacturer is 0.25 10^{-6} ml/grid, could you determine the number of yeasts per ml of suspension?

Grid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
No. cells	12	14	12	11	12	14	15	12	9	11	12	14	15	10	11	9	10	11	12	14

For several decades, **epifluorescence microscopy** has allowed the visualization and enumeration of microorganisms previously stained with fluorochromes. This is a technique valid for quantification of bacteria (Figure 4).

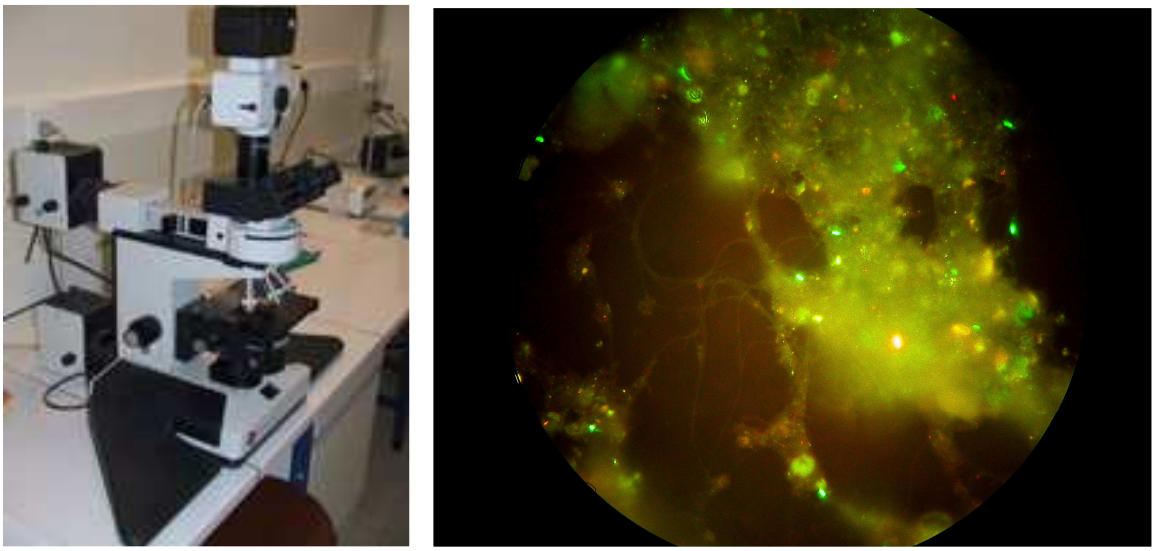


Figure 4. Epifluorescence microscope and image of a sample stained with acridine orange.

In order to determine the microbial density in a given sample it is necessary to know the microscope factor (number or fields per filter). The equation used to convert the data into a microbial density is the following one:

$$\text{Microorganisms/ml} = \frac{\text{Average number of microorganisms/field}}{\text{Volume filtered (ml)}} \text{ X Microscope factor X Dilution factor}$$

2.7. To estimate the bacterial density of a water sample, 100 µl of this sample was stained with acridine orange and filtered through a membrane filter of 0.2 µm pore diameter. The filter was mounted on a slide and observed using an epifluorescence microscope. 20 fields were counted and number of bacteria obtained by field was recorded in the following table:

Fields	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
No. cells	15	12	11	9	21	16	13	13	15	20	22	13	14	12	18	17	9	22	18	18

If the microscope factor is 30,954 fields/filter, what is the bacterial density of the sample? What would the bacterial density be if 1 ml of the 10^{-2} dilution had been filtered?

Finally, a classic and very simple method for microbial quantification is based on the **measurement of the absorbance** (at a given wavelength) of a microbial suspension and the transformation of this value into a number of cells (or CFU) per ml. Obviously, this method requires a previous study in which various dilutions of the microbial suspension are prepared and measured (absorbance) in order to get an equation that correlates both parameters. In future studies, this equation can be used to determine the microbial density from absorbance data.

2.8. From a dense suspension of *Escherichia coli* we have prepared different suspensions in which absorbance and CFU/ml were determined. The results obtained are given in the following table:

Suspension	**Absorbance (550 nm)**	**No. bacteria/ml**
1	0.020	$3.55\ 10^{6}$
2	0.052	$2.04\ 10^{7}$
3	0.102	$5.65\ 10^{7}$
4	0.164	$1.95\ 10^{8}$
5	0.213	$4.75\ 10^{8}$
6	0.264	$6.90\ 10^{8}$

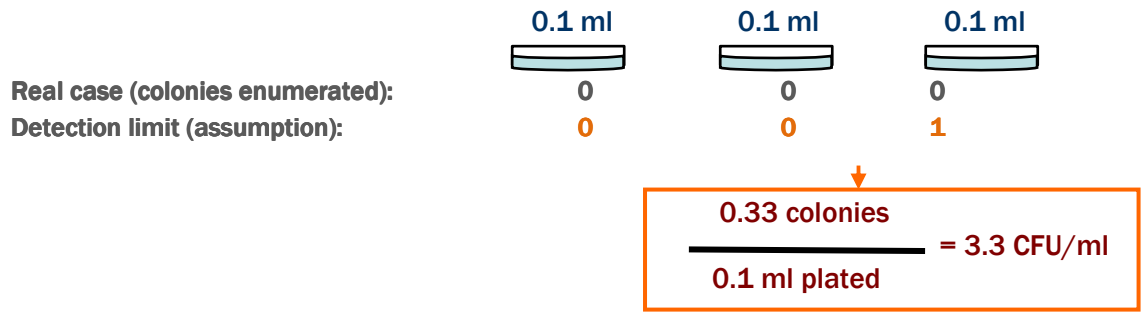
If we have a sample with an absorbance of 0.18, what is the bacterial density?

SOLUTIONS

2.1. CFU/ml? Volume spread = 100 µl/plate

Sample	Dilution	Plate 1	Pla7e 2	Plate 3	CFU/ml
1	10^{-3}	1816	1698	1885	
	10^{-4}	180	159	186	$1.75\ 10^{7}$
	10^{-5}	16	19	10	
2	10^{-2}	475	477	480	
	10^{-3}	45	48	51	$4.8\ 10^{5}$
	10^{-4}	5	10	4	
3	10^{0}	335	328	324	
	10^{-1}	32	28	29	$2.97\ 10^{3}$
	10^{-2}	5	3	2	

2.2. Detection limit for CFU method: 100 µl/plate, 3 plate/sample



Detection limit (assumption): 1 colony/3 plates = 3.3 CFU/ml

Real case: 0 colonies/3 plates = Detection limit = <3.3 CFU/ml

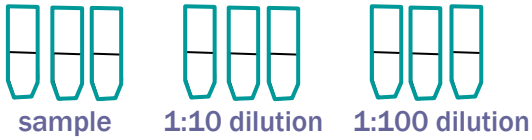
Detection limit for this situation: 100 µl /plate, 5 plates/sample

Detection limit = <2 CFU/ml

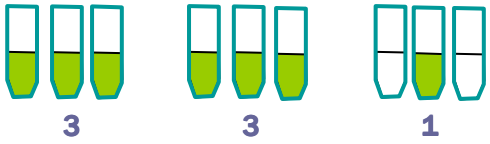
2.3. Microdrop method (10 µl/drop). CFU/ml?

Dilution	Count 1	Count 2	Count 3	CFU/ml
10^{-3}	180	159	186	
10^{-4}	16	19	10	$1.5\ 10^{7}$

2.4. MPN. Bacterial density?

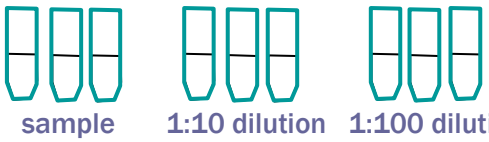


Inoculated volume = 1 ml of sample or dilution
Incubation: 48 h at 20ºC
Check growth (turbidity)



45 bacteria/ml

2.5. MPN. Detection limit?



Real case (tubes positives):	0	0	0
Detection limit:	1	0	0

Detection limit <0.3 bacteria/ml

0.3 bacteria/ml

2.6. Yeast suspension. Diluted 1,000 times. Chamber Factor = 0.25 10^{-6} ml/grid
No. yeasts/ml of suspension?

Grid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
No. cells	12	14	12	11	12	14	15	12	9	11	12	14	15	10	11	9	10	11	12	14

$$\frac{12 \text{ cells/grid}}{0.25\ 10^{-6} \text{ ml/grid}} \times 1{,}000 = 4.8\ 10^{10} \text{ yeats/ml}$$

2.7. Epifluorescence microscopy. Volume filtered = 100 µl. Microscope Factor = 30,954 fields/filter. Bacterial density?

Field	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
No. cells	15	12	11	9	21	16	13	13	15	20	22	13	14	12	18	17	9	22	18	18

$$\frac{15.4 \text{ bacteria/field} \times 30{,}954 \text{ fields/filter}}{0.1 \text{ ml/filter}} = 4.77\ 10^6 \text{ bacteria/ml}$$

Volume filtered = 1 ml. Dilution 10^{-2}. Microscope Factor = 30.954 fields/filter. Bacterial density?

$$\frac{15.4 \text{ bacteria/field} \times 30{,}954 \text{ fields/filter} \times 100}{1 \text{ ml/filter}} = 4.77\ 10^7 \text{ bacteria/ml}$$

2.8. Density of a suspensión of *E. coli* with an absorbance (550) of 0.18?

Suspension	Absorbance (550 nm)	Bacteria/ml	Log bacteria/ml
1	0.020	$3.55\ 10^6$	6.55
2	0.052	$2.04\ 10^7$	7.31
3	0.102	$5.65\ 10^7$	7.75
4	0.164	$1.95\ 10^8$	8.29
5	0.213	$4.75\ 10^8$	8.68
6	0.264	$6.90\ 10^8$	8.84

Log bacteria/ml = 8.982 Abs + 6.683 (r = 0.97)

$1.994\ 10^8$ bacteria/ml