ENUMERATION OF MICROORGANISMS

I. OBJECTIVES

- To learn the different techniques used to count the number of microorganisms in a sample.
- To be able to differentiate between different enumeration techniques and learn when each should be used.
- To have more practice in serial dilutions and calculations.

II. INTRODUCTION

For unicellular microorganisms, such as bacteria, the reproduction of the cell reproduces the entire organism. Therefore, microbial growth is essentially synonymous with microbial reproduction. To determine rates of microbial growth and death, it is necessary to enumerate microorganisms, that is, to determine their numbers.

It is also often essential to determine the number of microorganisms in a given sample. For example, the ability to determine the safety of many foods and drugs depends on knowing the levels of microorganisms in those products. A variety of methods has been developed for the enumeration of microbes. These methods measure cell numbers, cell mass, or cell constituents that are proportional to cell number. The four general approaches used for estimating the sizes of microbial populations are direct and indirect counts of cells and direct and indirect measurements of microbial biomass. Each method will be described in more detail below.

1. Direct Count of Cells

Cells are counted directly under the microscope or by an electronic particle counter. Two of the most common procedures used in microbiology are discussed below.

Direct Count Using a Counting Chamber

Direct microscopic counts are performed by spreading a measured volume of sample over a known area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Specially constructed counting chambers, such as the Petroff-Hauser and Levy counting chambers, simplify the direct counting procedure because they are made with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area

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and convert the numbers observed directly to volume makes the direct enumeration procedure relatively easy.

Direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. This method is used to assess the sanitation level of a food product and in performing blood cell counts in hematology. The differential white blood cell count, which is used as an indication of the nature of a microbial infection, involves direct counting of blood cells that have been stained to differentiate different types of white blood cells.

Direct Count Using Fluorescent Dyes

Fluorescent dyes are becoming more used in recent years for a variety of procedures, one of which is bacterial counts. These dyes can be employed to stain all species, a particular species of interest in an environmental sample or even a specific component of cells.

The most widely used fluorescent dye for counting the number of bacterial cells is acridine orange which stains both living and dead cells by interacting with DNA and protein components of cells. The stained cells fluoresce orange when excited near ultraviolet light. This stain is particularly useful for determining the total number of microorganisms in samples, such as soil and water, where the co-existence of metabolically diverse populations precludes establishing conditions for the simultaneous enumeration of microbial populations by viable count procedures. The procedure is widely used in marine microbiology where population levels are often low and where viable plate counts are known to severely underestimate total number of bacteria. Typically, the viable count is less than 1% of the direct count for marine samples.

In this procedure the bacteria in a known volume of sample are stained with acridine orange and the sample is then filtered through a 0.22 μm filter. The bacteria are trapped on the filter that is then examined under a fluorescence microscope. The bacteria in a defined area of the filter are counted and the concentration in the original sample is then calculated.

Other fluorescent dyes that are also gaining popularity are cyanoditolyl tetrazolium chloride (CTC), auramine and rhodamine. CTC binds to respiration proteins in the cell and thus can demonstrate live cells. Auramine and rhodamine bind to cell wall of Mycobacteria and emit bright yellow or orange color under a fluorescent microscope. These latter stains are gradually replacing the acid-fast stain.

2. Indirect Count of Cells

Microorganisms in a sample are diluted or concentrated and grown on a suitable medium; the development of growing microorganisms (for example, colony formation on agar plates) is then used to estimate the numbers of microorganisms in the original sample.

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Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the "colony forming units". One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material.

Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} (1/10) to 10^{-8} (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10^{-6} because we know that if there were 10^{7} or more bacteria per milliliter, the water would be turbid.

The Most Probable Number (MPN)

The most probable number procedure dates back to the earliest days of microbiology. However, it is still widely used in sanitary bacteriology to estimate numbers of coliforms in water, milk, and other foods. Coliforms are bacteria that reside in the intestine of warm-blooded mammals and are regularly excreted in the feces. They are Gram negative rods belonging to the *Enterobacteriaceae* family, ferment lactose and produce gas. Not all members of *Enterobacteriaceae* are coliforms.

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The MPN procedure is a statistical method based upon the probability theory. Samples are serially diluted to the point of extinction, that is, to a point where there are no more viable microorganisms. To detect the end point, multiple serial dilutions are inoculated into a suitable growth medium, and the development of some recognizable characteristic, such as acid production or turbidity, is used to indicate growth (the presence of at least one viable microorganism in the diluted sample). The pattern of positive tests (growth) in the replicates and statistical probability tables are used to determine the concentration (most probable number) of bacteria in the original sample. Statistical MPN tables are available for replicates of 3, 5, and 10 tubes of each dilution. The more replicate tubes used, the greater the precision of the estimate of the size of the bacterial population.

In this exercise, we will use a three-tube MPN procedure to estimate the numbers of coliforms in a water sample. As the positive criterion for identifying coliforms, we will use the ability to ferment lactose with the production of acid and gas; acid production will be detected using bromcresol purple as a pH indicator (the change from purple to yellow = acid production) and gas production will be detected using inverted Durham tubes.

3. Direct Measurement of Microbial Biomass

Cell mass is determined directly by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of bacteria may be used for estimation of cell numbers.

4. Indirect Measurement of Microbial Biomass

Microbial biomass is estimated by measuring relatively constant biochemical components of microbial cells, such as protein, ATP, lipopolysaccharides, peptidoglycan, and chlorophyll; biomass can also be indirectly estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve.

Various procedures based on the detection of specific microbial macromolecules or metabolic products can be used to estimate numbers of microorganisms. For example, peptidoglycan can be quantified, and because this biochemical occurs exclusively in the cell wall of bacteria, the concentration of peptidoglycan can be used to estimate bacterial numbers. Such biochemical approaches for determining bacterial numbers depend on the development of analytical chemical procedures for quantifying the particular biochemical and determining what proportion of bacterial cell is composed of the specific biochemical constituent.

III. LABORATORY SUPPLIES

Viable Plate Count

Test sample, 2 ml/test tube	1/group
BHI plates, dried at 37°C	6/group
Saline, 9.9 ml/16 mm test tube	4/group
Pipettes, 1 ml	4/group

Direct Count Using a Counting Chamber

Yeast suspension	2 ml/lab
Counting chamber	1/student
Pipettes, Pasteur	1/student

Most Probable Number Method

Test sample	same as viable count
Lactose-bromcresol purple broth	9/group
Saline tubes	same as viable count
Pipettes, 5 ml	5/group

IV. PROCEDURES (Direct count is done by each student individually; however, the students at a table will form a group to perform the viable count and the MPN.)

First Session

Direct Count Using a Counting Chamber

1. Clean a counting chamber with methanol and lens paper and then place it on the microscope stage.

2. Using the 4X objective find the ruled area on one side of the chamber and note the size and arrangements of larger squares and their small square subdivisions.

3. Shake the yeast suspension to distribute the cells evenly. Take out the counting chamber without changing the focus on the 4X objective. Place a coverslip over the calibrated surface of the counting chamber.

4. Using a transfer pipette, transfer some of the yeast suspension to the groove of the counting chamber to fill the chamber by capillary action.

5. Carefully place the counting chamber back onto the microscopic stage and observe the cells under 4X. You may need to reduce the amount of light by closing the diaphragm of the condenser to be able see the cells

6. Switch to the high-dry objective (40 X) and count the number of yeast cells in at least 50 of the small squares. If cells fall on a line, include in your count those on the top and left-hand lines and exclude those on the bottom and right-hand lines. (If the yeast cells are too dense to count, dilute your sample and start again.)

7. Calculate the average number of yeast cells per small square. Then calculate the number of yeasts per ml by dividing the average number of yeasts per small square by the volume of each small square which is 0.00025 μl. If you diluted the sample you must also multiply your results by the dilution factor to determine the

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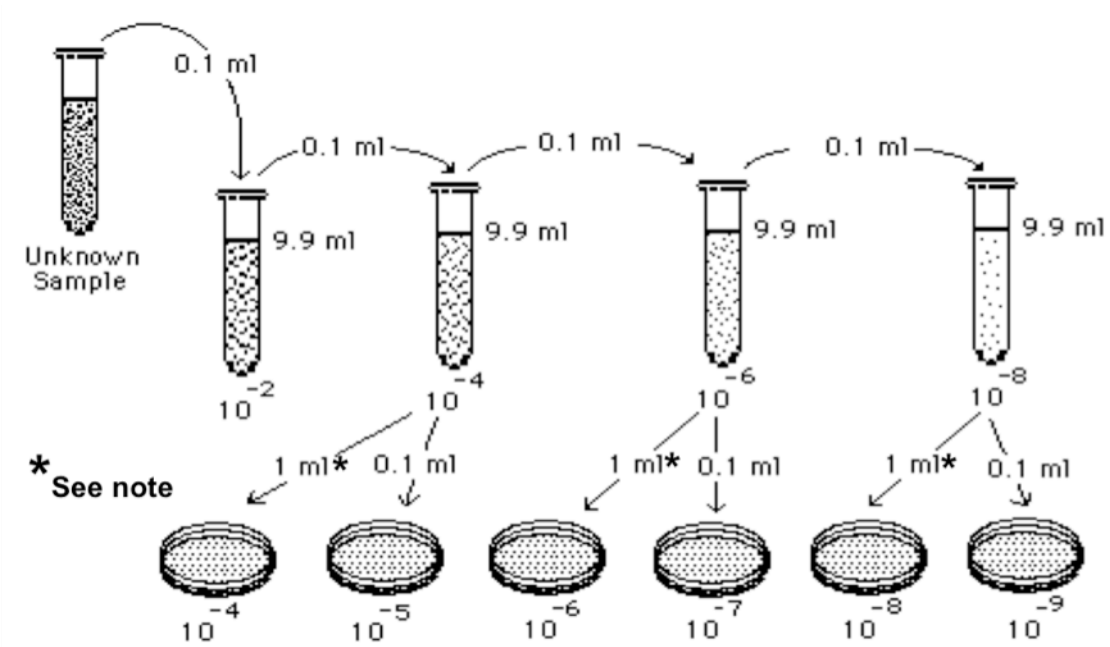
concentration of yeast cells in the original sample. Record your calculations and results.

Viable Plate Count

1. Label four 9.9 ml saline tubes 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}, respectively. Label six BHI plates 10^{-4} to 10^{-9}.

2. Vortex the unknown sample to ensure an even distribution of bacteria. Aseptically remove 0.1 ml of sample with a sterile pipette and transfer it to the 10^{-2} dilution tube (see diagram).

3. Vortex the 10^{-2} tube and transfer 0.1 ml to the 10^{-4} tube.



4. Again vortex the 10^{-4} dilution tube and transfer 0.1 ml to the 10^{-6} tube. Vortex this last tube well.

5. Vortex the 10^{-6} tube, transfer 0.1 ml to 10^{-8} tube and vortex again.

6. Using a new sterile pipette, aseptically transfer 1.0 ml from the 10^{-4} dilution tube to the plate labeled 10^{-4} and 0.1 ml to the plate labeled 10^{-5}. Spread the inoculum on the surface of the agar in each plate using an alcohol-dipped, flamed, metal spreader. Dip the spreader into the alcohol jar and quickly take it through the flame and let the alcohol burn off after each spreading. Do not allow the spreader to get too hot. Never hold the spreader in the flame for more than a second.

Note: (See stars on the figure above) Since it would take long for the agar in the petri dish to absorb the 1 ml sample, if the dish is moved during this time, it is possible to obtain wrong results as the dividing cells may move to different parts of the plate and produce their own colonies. To prevent this, place the 1.0 ml samples in Eppendorf tubes, microfuge the tubes for 1 minute, discard about 0.9 ml of supernatant, resuspend the pellet in the liquid left and spread the contents on their appropriate plates.

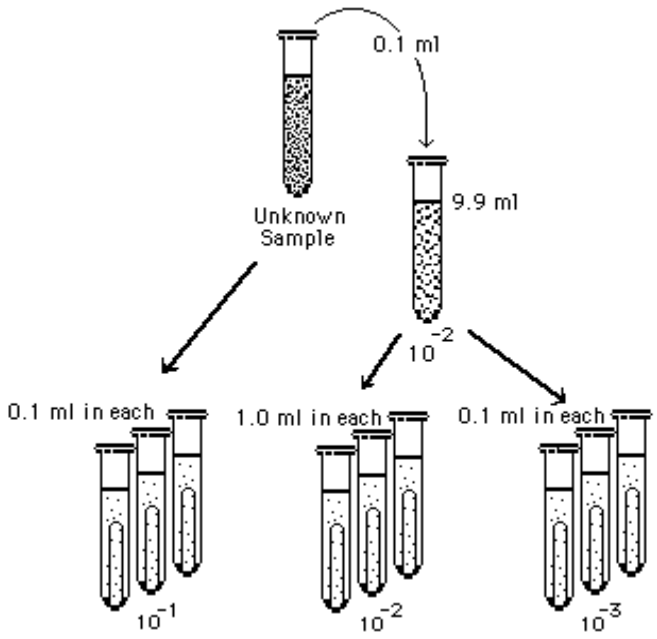
Important Note: To sterilize the spreader, dip it in the 95% ethanol jar, shake the extra alcohol off by touching the inside of the jar above the alcohol level and then quickly take the spreader through a flame. Make sure that you do not hold the spreader in the flame for more than a second. It is the alcohol and not the heat that kills any bacteria present. Be extra careful as flaming alcohol drops may fall on objects and cause a fire hazard. Also while the alcohol is burning off, keep the spreader head down so the dripping alcohol will not spread to your fingers.

7. Repeat the above to transfer 1.0 and 0.1 ml from the 10^{-6} dilution tube to the 10^{-6} and 10^{-7} plates, respectively. In the same manner establish the 10^{-8} and 10^{-9} plates. Do not discard your dilution tubes. (See note above)

8. Allow the surface of the agar to dry before you move or invert the plates. Incubate the plates at 37°C for 2 days.

Most Probable Number (MPN) Method

1. Label 9 lactose-bromcresol purple tubes for the following dilutions in triplicate: 10^{-1}, 10^{-2} and 10^{-3}.



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2. Using a 5 ml pipette, take 3.3 ml of 10^{-2} dilution tube from the viable count procedure and dispense 0.1 ml into each of the 10^{-3} MPN tubes and 1.0 ml into each of the 10^{-2} MPN tubes. Use the same pipette to take 0.3 ml of the original sample and distribute 0.1 ml into each of the 10^{-1} MPN tubes. Mix contents of tubes very gently not to disturb the Durham tubes inside.

3. Incubate the tubes at 37°C until the next laboratory session.

Second Session

Viable Plate Count

1. After incubation, count the colonies on each of the plates. Holding the plate to a light source, count the colonies by marking their position on the back of the petri plates with a marking pen. This aids in keeping track of those colonies previously counted and avoids recounts. If a plate has more than 300 colonies, record it as TNTC (too numerous to count).

2. From the plate count data, calculate the concentration of bacteria in the original sample. For statistical reasons use only data from plates which have between 30 and 300 colonies in this calculation. Each colony forming unit (cfu) represents a single cell or a group of cells attached together and inseparable by shaking. Therefore, the number of cfu in the original sample is determined by multiplying the number of colonies on a dilution plate by the corresponding dilution factor. For example, if there are 200 colonies on the 10^{-4} plate, then there are 200 x10,000 = 2,000,000 colonies or 2 x 10^6 cfu/ml in the original sample. Generally replicates of each dilution are plated, and the mean count is recorded. Thus the mean of data from all groups in the lab would be an excellent estimate of the number of bacteria in the original sample.

Most Probable Number (MPN) Method

1. After incubation, examine tubes for the presence of BOTH acid and gas. Use the following MPN table to record the most probable number of coliforms in your sample.

MPN TABLE

MPN values & 95% Confidence limits for various combinations of positive results, when three replications of each sample are used.

Number of tubes giving positive reaction out of 3 for the following dilutions			MPN value	95 % Confidence Limits	
10^{-1}	10^{-2}	10^{-3}	per ml	Lower	Upper
0	0	1	3	0.5	9
0	1	0	3	0.5	13
1	0	0	4	0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

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Results of the Bacterial Enumeration Exercises

NAME _____ DATE _____ GROUP NAME _____

NAME OF PARTNER(S) _____

Viable Count

Dilution	Count
10^{-4}	
10^{-5}	
10^{-6}	
10^{-7}	
10^{-8}	
10^{-9}	

Estimate of the cell concentration in the original stock =

The most Probable Number (MPN) Method

Number of positive lactose-bromcresol purple tubes at 10^{-1} dilution:
Number of positive lactose-bromcresol purple tubes at 10^{-2} dilution:
Number of positive lactose-bromcresol purple tubes at 10^{-3} dilution:

Most probable number of coliforms =

Lower and upper 95% confidence limits =

Direct Counts Using A Counting Chamber

Total number of yeast cells counted _____

Average number of yeast cells per counting squares _____

Volume associated with each counting square _____

Dilution factor (if any)_____

Concentration of yeasts in original sample_____

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