

Dilution plating

Background information

Dilution plating is a simple technique used to estimate the number of heterotrophic bacteria in an environmental sample. The technique can also provide an index by which differing environments or treatments can be compared. For example, because the number of bacteria in environmental samples is affected by many variables including the availability of usable substrates, the presence of contaminants (organics or metals) and/or physical disturbance, cell counts can be used to compare disturbed/contaminated environments with pristine ones.

Dilution plating is based on serial dilution of the sample. Serial dilution involves repeatedly mixing known amounts of source sample or culture with sterile liquid, usually a low-molarity buffer. In such a scheme, 1 ml of sample added to 9 ml of buffer to yield a 10-fold dilution (1:10, or 10^{-1}); 1 ml of the 10-fold dilution is added to another 9 ml of buffer to yield a 100-fold dilution (1:100, or 10^{-2}), etc. When fixed volumes of this dilution series (e.g. 100 μ l of each of the dilutions) are spread onto a solid growth medium and incubated, different numbers of colonies will be obtained. By noting the number of colonies, the volume of inoculant added, and the mass or volume of sample diluted, one can calculate the number of microorganisms in the original sample.

Since bacteria are cultured on defined carbon sources, dilution plating detects for a subpopulation of the whole community. Therefore, using different media (carbon/nitrogen sources) will likely give differing results. Despite this limitation, dilution plating can provide a good initial estimation of the number of bacteria in a given community. These limitations can be avoided by performing direct cell counts. Direct cell counting is culture independent, involving staining and microscopic analysis of bacterial cells. Although this method is highly accurate it can be time consuming and requires some expensive equipment and considerable training to generate consistent results.

Materials

10 mM phosphate buffer (pH 7, sterile)
Nine Petri dishes for each media (in this lab we will use R2A medium)
Six - 15 ml Falcon tubes
One - 200 ml milk dilution bottle containing 100 ml of sterile buffer

Ethanol
Bunsen burner
Inoculant spreader
200 and 1000 μ l pipettes with tips
Metal spatula

- Wear gloves throughout the entire protocol.
- Do not cross-contaminate your samples OR THE SOLUTIONS. Be aware of your pipette tip.
- Work clean, either on fresh blue bench paper, in the hood, or on a freshly ethanol treated bench top.
- Do not use a vortex at any point in this protocol unless specified.

The protocol in brief

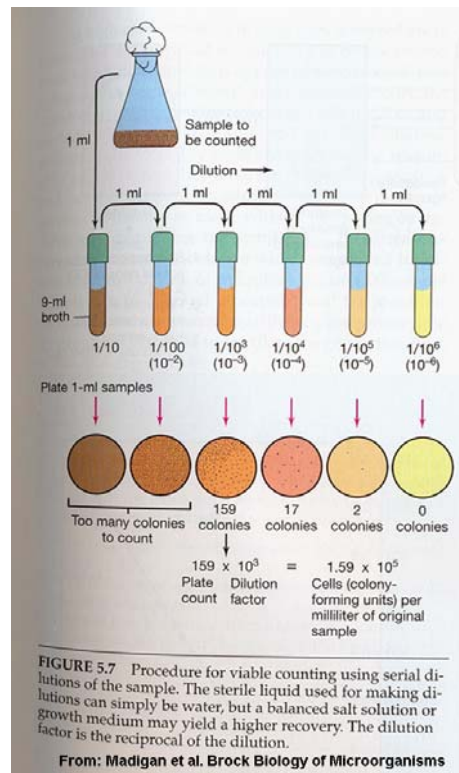
You will estimate the number of heterotrophic bacteria in your sample by diluting your sample, plating it onto solid nutrient media, incubating your plates, and then counting the number of bacterial colonies that appeared. Counts of the bacterial colonies (colony forming units) resulting from the appropriate dilution will allow you to calculate (estimate) the number of heterotrophic bacteria in your sample.

A. Diluting samples (for low biomass water samples see p. 5 of this protocol)

Soil, plant material or sediment

1. Weigh 5 g of material into a 200 ml milk dilution bottle containing 100 ml of sterile 10 mM phosphate buffer and shake the bottle by hand approximately 50 times (best method). Alternatively, weigh the soil into a 50 ml falcon tube, add 35 ml of buffer, then vortex the tube for approximately one minute.
2. Shake the bottles/tubes in a reciprocating shaker for 30 minutes at the highest setting (200 rpm).
3. Remove the tubes from the shaker and allow the suspension to settle for 15 minutes.
4. Fill each of six - 15 ml falcon tubes with 9 ml of sterile buffer and label each tube to reflect a ten-fold dilution of the previous tube (tube 1 = 10^{-1} , tube 2 = 10^{-2} , etc.). Write the dilution of the sample on each tube.

- Using a 1 ml pipette, transfer 1 ml of the suspension from the sample tube to the first dilution tube (10^{-1}). Briefly vortex the 10^{-1} tube.
- Transfer 1 ml of the suspension from the 10^{-1} dilution tube to the second dilution tube (10^{-2}). Briefly vortex the 10^{-2} tube.
- Repeat steps 5 - 6 until the original sample is diluted to 10^{-6} (figure below). Proceed to section "B", *Plating the samples*.



Moderate to high biomass water samples (e.g. lake-, waste-, and pond water)

- Fill each of six - 15 ml falcon tubes with 9 ml of sterile buffer and label each tube to reflect a ten-fold dilution of the previous tube (i.e. tube 1 = 10^{-1} , tube 2 = 10^{-2} , etc.). Write the name of the sample on each tube.
- Using a 1 ml pipette, transfer 1 ml of water sample to the first dilution tube (10^{-1}). Briefly vortex the 10^{-1} tube.
- Transfer 1 ml of the suspension from the 10^{-1} dilution tube to the second dilution tube (10^{-2}). Briefly vortex the 10^{-1} tube.
- Repeat steps 2 - 3 until the original sample is diluted 10^{-6} . Proceed to section "B", *Plating the samples*.

B. Plating the samples

Samples from three consecutive dilutions should be plated in triplicate to ensure that countable plates (between 30 – 300 colonies) will be generated. Samples will be plated beginning with the lowest dilution. Therefore, the same pipette tip can be used for each of the nine inoculated plates (three dilutions x three replications). Between pipettings, place the pipette on the bench surface with the tip extended over the edge of the bench top. Avoid touching the tip to any surface. If the tip contacts any surface, replace it with a new one.

8. Transfer 100 μl of the highest dilution to be plated to the surface of each of three Petri dishes containing sterile agar media. A rapid method of inoculation will be demonstrated in class.
9. Sterilize a glass spreader by soaking its spreading surface in 70 % ethanol and pass it through a flame one time. The alcohol will kill any bacteria on the glass surface and the flame will burn-off the alcohol.
10. After the flame has extinguished, remove the lid from the Petri dish. Touch the spreader to the agar surface to cool the glass. Do not touch the hot glass to the added cell suspension or you will kill some of the cells. Using a back-and-forth motion, spread the inoculant over the surface of the agar. Replace the lid of the Petri dish.
11. Repeat steps 8 – 10 for the next two lower dilutions.

C. Incubation and counting

12. Incubate the plates at 25° C for 24-48 hours.
13. After the incubation is complete, identify the dilution that resulted in the growth of between 30 – 300 bacterial colonies. Count the colonies and write the colony number on the plate.
14. Determine the average number of colonies on the three counted plates.
15. Using the following formulae, determine the number of bacteria in the original sample based on the average number colonies determined in step 14:

$$\text{Number of colonies ml}^{-1} \text{ of undiluted, suspended sample or water} = \frac{AVE \times DIL}{VOL}$$

, where

AVE is the average number of colonies on the three counted plates

DIL is the dilution factor of the Petri dishes that were counted
VOL1 is the inoculated volume (0.1 ml)

For solid samples, such as soil, plant material or sediment, an additional calculation is necessary to express the number of bacteria on a per gram basis;

Number of colonies g^{-1} of suspended sample (wet weight) = $\frac{NUM \times VOL2}{MASS}$, where

NUM is the number of colonies per ml of suspended sample (calculated above)

VOL2 is volume of the original suspension (100 ml)

MASS is the mass (g) of solid material added to the original suspension

To relate the bacterial number to the dry weight of material, simply multiply the number of colonies g^{-1} wet weight times the % water in the sample (decimal equivalent) and subtract this value from the number of colonies g^{-1} wet weight.

Low biomass water samples (e.g. drinking water, pristine sources such as streams and oligotrophic lakes)

Low biomass water samples require a concentration step, rather than dilution, prior to plating. Therefore, the following steps outline a filtration procedure that traps bacteria on a membrane thereby concentrating them from a large volume of water. The membrane is then incubated atop agar media and bacterial numbers are estimated in the same manner as above.

Additional materials needed

Millipore filtration unit

Vacuum manifold

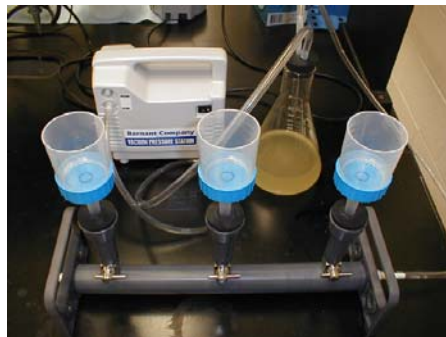
Sterile forceps

0.45 μm polycarbonate membrane filter with absorbent pad

47 mm Petri dish with appropriate media

1. Sterilize forceps by soaking in 70% ethanol and passing through a flame. The alcohol will kill any bacteria on the glass surface and the flame will burn-off the alcohol. With sterile forceps, remove a filter membrane (0.45 μm) and absorbent pad from plastic wrapping and place it onto the base of a Millipore disposable filtration unit. Be sure to place the pad-side down with the filter facing up. Snap the filter cup onto the base.

2. Place the filtration unit onto a vacuum manifold. Open all of the valves that control flow to vacuum ports that have filtration units attached. Close all others.
3. Place an appropriate volume of sample into the cup. A good starting point is 100 ml. Depending on the number of bacteria in your samples, 1000 ml and 10 ml volumes can be filtered as well.
4. Hook up the tubing from the vacuum pump to the manifold via the back-flow preventor. Turn the pump on. The water will be sucked through the filter membrane while cells present in the water will be trapped on the membrane.



The vacuum manifold (gray) with three filter units attached. A vacuum pump pulls solutions through the filters and collects the filtrate in a backflow preventor made from an Erlenmeyer flask.

5. Close the valves after each water sample has completely passed through each membrane to increase the vacuum suction to the remaining samples. Turn off the pump when each of the water samples has passed through the membranes.
6. Open the valves to release any remaining suction. Remove the filtration units from the vacuum manifold and remove the filtration cup from that base without disturbing the membrane.
7. With sterile forceps, carefully separate the membrane from the pad/base and place (bacteria-side “up”) into a Petri dish containing solid media.
8. Replace the lid of the dish and incubate samples and go to section “C” above for incubation and counting instructions.