Lab #2, Soil Bacteria and Fungi
Forests Through Time and Space Winter 05

Objectives

- Estimate the number of colony-forming units (CFUs) of bacteria and actinomycetes per gram soil.
- Become familiar with serial dilutions and spread plating.
- Calculate the number of CFUs per gram soil using spread plate counts
- Become familiar with the macroscopic and microscopic features of soil bacteria and fungi

What to Bring to Lab

- Soil sample (app 50 g)

Background

There are a number of different methods for determining the number of microbial colony-forming units (CFUs) in soil. The results depend on diluting the CFUs to a concentration where the growth of one colony does not inhibit neighboring colonies. Bacteria are the most numerous culturable organisms in soils (viruses are more numerous, but difficult to culture—you need a suitable host). The main species are *Arthrobacter, Bacillus, Pseudomonas,* and *Streptomyces*. *Arthrobacter,* and *Streptomyces* are actinomycetes which produce cells that resemble fungi. The results you will get depend on the media used. Media for isolating bacteria is usually nutrient poor to inhibit the growth of fungi, while that used for fungi often has antibiotics and is acidified to inhibit bacterial growth.

There are a number of potential problems with evaluating the results of these assays in terms of what they mean in the soil. Nevertheless, these techniques are still commonly used to compare soils. Think carefully about the assumptions that lead to the numbers you get. You will be asked to speculate on this in your report.

Methods

Serial Dilution (week 1)

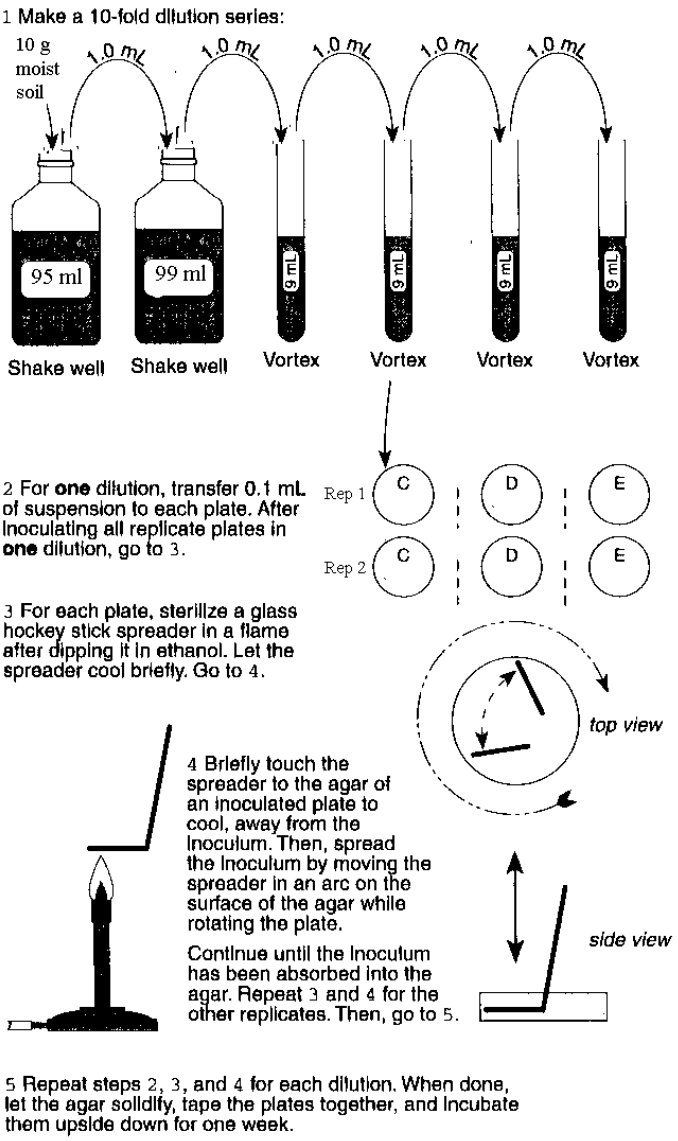
Materials—1 sterile 95 ml water blank, 1 sterile 99 ml water blank, 5 sterile 9 ml water blanks, 6 sterile 1 ml pipettes

1. Label five 9 ml water blanks with 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7},and 10^{-8}.
2. Weigh out 10.0 g of moist soil (record the exact weight) and put into a 95 ml water blank. Cap and shake well (about 10 minutes).
3. Using a sterile pipette, transfer 1.0 ml of the original suspension into the 99 ml water blank (creating the 10^{-3} dilution). Cap and shake well (about 5 minutes).
4. Using a new sterile pipette, transfer 1.0 ml of the above suspension to the first 9 ml water blank (10^{-4}). Cap and shake or vortex well (3–5 minutes).
5. Repeat step 8 using the 10^{-4} dilution as the source and transferring 1.0 ml into the 10^{-5} blank. Repeat for all subsequent dilutions (Fig 1).

Inoculation of spread plates (week 1)

Materials—six plates each of tryptic-soya agar and Martin's Rose Bengal agar, alcohol lamp, glass hockey stick, jar of 95% EtOH to dip hockey stick, 3 sterile 0.1 ml pipette tips and pipettor, 2 plastic bags to hold plates (Fig 1).

6. Label two plates of each media on the bottoms with the media type, dilution, and your names for the **three most dilute solutions (10^{-6}, 10^{-7},and 10^{-8}).**
7. Beginning with the most dilute soil suspension, shake and, using a sterile pipette, transfer 0.1 ml to each the four plates for that dilution (1 of each medium) (Fig 1).
8. Dip the hockey stick into the EtOH to sterilize it and lightly flame it to remove the EtOH. Allow to cool briefly.
9. Briefly touch the hockey stick to the agar to cool in an inoculated plate away from the inoculum (don't want to cook the inoculum). Spread the inoculum by moving the hockey stick in an arc while rotating the plate. Continue until all the water has been absorbed into the agar. Repeat steps 16 and 17 for the other plate for this dilution.
10. Repeat steps 15–17 for the other two lowest dilutions.
11. Put the plates in a plastic bag (all one medium together) and incubate the plates inverted for one week for the bacteria. Paul will check on the plates and put them in the cooler when they've grown sufficiently



Observation and Enumeration (week 3)

Materials—inoculated plates from last week , counter, marker, inoculating loop, ethanol, microscope slides, alcohol lamp, compound scope, immersion oil.

In order to get an accurate count, you need between 20–200 colonies per plate. Plates that fall outside of this range won't give accurate estimates. Observe not only the quantity, but also the qualitative aspects of the colonies. Bacteria generally appear smooth and creamy, or mucoid. They can be discrete round colonies, spreading or feathery. Actinomycetes typically have a white, gray, or black powdery surface, and are firm and leathery. Actinomycete colonies will break under pressure and resist movement when scraped with a needle while bacteria will smear. A clear zone indicative of antibiotic production often surrounds actinomycetes.

12. Count the appropriate dilution plates for each medium. Record the numbers of bacteria & actinomycetes on the tryptic-soya agar and the number of fungi and actinomycetes on the Martin's Rose Bengal agar.
13. Observe representatives of the various microorganisms through the compound microscopes as outlined below.

14. Transfer a small drop of tap water for a slide with an inoculating loop. Flame the loop and remove a small amount of culture. Mix the bacteria in the drop of water, spreading it over an area about the size of a dime.
15. Cover with a cover slip and examine first at 400x (40x objective)
16. Examine the slide using oil and the immersion objective. Describe and sketch what you see.
17. To examine fungi, remove a small bit of mycelium (can include a little agar too) from a fungal colony using a dissecting needle as follows:
    a. Flame the needle, quench, and flame off EtOH.
    b. Slowly open petri dish just enough to get needle into colony, open the dish away from you, or to the side.
    c. Cut a small bit of mycelium and transfer it to a slide.
    d. Add a half drop of KOH and a half drop of phloxine, mix with mycelium, put coverslip on and gently squish. If you label one end of your slide, you can do two or three specimens per slide.
    e. Examine the slide using the 40X objective, looking for clamp connections (ask if you have any doubts). Most of the white-rot basidiomycetes, as well as other basidiomycetes, have clamp connections between adjacent "cells" in the mycelium. Record all your observations with sketches.
18. Repeat the process in 17 for any suspected Actinomycetes. Sketch and describe what you see.

Calculations

You have the data to estimate the number of CFUs per gram dry soil for bacteria/actinomycetes and fungi from the spread plates.

Spread Plate Calculations

1. Calculate the grams of soil per ml in your original solution by dividing the weight of the soil used by 100 ml.
2. Average the counts for the appropriate dilution/medium combination. Use only one dilution for your calculations. Average the counts for the two plates
3. Calculate the average CFUs per gram soil as follows. Average CFUs ÷ 0.1 ml ÷ dilution ÷ grams of soil per ml. To continue our example, let's say we got 154 CFUs per plate at the dilution of 10^{-4}. Our final CFUs per gram soil would be: 154 CFUs per plate ÷ 0.1 ml per plate ÷ $1x10^{-4}$ ÷ 7.48 x 10^{-2} g soil per ml = 2.06 x 10^{8} CFUs/ gram dry soil.

Questions

Summarize all your data into neat tables and compare your data with two other groups. Answer the following questions in your discussion.

1. How did the abundance of bacteria and fungi vary between sites? How do you explain the differences/similarities?
2. What assumptions are inherent in the method you used? Are they valid?
3. What do the results really mean? Do they reflect the activity of these microbes in the soil? Why or why not?
4. How could you determine the number of actively growing CFUs in the soil?
5. If you were to repeat these experiments, what might you do differently?