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Visualizing Bacterial Colonies on Agar Plates: Dilution and Plating of Soil to Allow for Bacterial Enumeration

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Environmental Science Education Title: Visualizing Bacterial Colonies on Agar Plates: Dilution and Plating of Soil to Allow for Bacterial Enumeration

Overview:

Surface soils are a heterogeneous mixture of inorganic and organic particles that combine together to form secondary aggregates. Within and between the aggregates are voids or pores that visually contain both air and water. These conditions create an ideal ecosystem for bacteria, so all soils contain vast populations of bacteria, usually up to 1000 million per grams of soil.

Bacteria are the simplest of microorganisms, known as prokaryotes. Within this prokaryotic group, there are the filamentous microbes known as actinomycetes. Bacteria and actinomycetes are found in every environment on Earth, but the abundance and diversity of these microbes in soil is unparalleled. These microbes are also essential for human life and affect what people eat, drink, breathe, or touch. In addition, there are bacterial species that can infect people and cause disease, and there are bacteria that can produce natural products capable of healing people, including antibiotics such as streptomycin from actinomycetes. Bacteria are critical for nutrient cycling, plant growth, and degradation of organic contaminants.

Bacteria are highly diverse in terms of the number of species that can be found in soil, in part because they are physiologically and metabolically diverse. Bacteria can be heterotrophic, meaning they utilize organic compounds, such as glucose, for food and energy, or autotrophic, meaning they utilize inorganic compounds, such as elemental sulfur, for food and energy. They can also be aerobic, utilizing oxygen for respiration, or anaerobic, utilizing combined forms of oxygen, such as nitrate or sulfate, to respire. Some bacteria can use oxygen or combined forms of oxygen and are known as facultative anaerobes.

One way to enumerate the number of bacteria present in a soil sample is to utilize dilution and plating methodology. This methodology utilizes agar as a medium for bacterial growth, a process termed, “culturable technology.” Because of the vast numbers of bacteria found within soils, a small sample of soil is serially diluted in water, prior to being plated on agar within a petri plate. Typically, a small amount of soil contained within 0.1 to 1 ml of the diluted soil suspension is “spread” over the surface of the agar plate. The plates contain agar, which is molten when hot, but solid when cool. In addition to the agar, nutrients, such as peptone yeast or a product commercially available as R_2A, are added to the medium to allow for the growth of heterotrophic bacteria.

Dilution and plating is an inexpensive and relatively simple technology for the enumeration of soil bacteria. However, there are several drawbacks to the technique. Some common errors and assumptions associated with dilution and plating assays are as follows: it is assumed that every single soil bacterium gives rise to a colony, but in reality a colony may arise from a clump of

cells, resulting in an underestimation of true culturable count. During serial dilution of the soil, soil particles can settle out (fall to the bottom), so the true aliquot of soil is not passed on into the next dilution. Many soil microbes are viable but non-culturable. Slow growing bacteria may not result in visible colonies within a reasonable time frame (1-2 weeks).

Also, anaerobic bacteria do not grow under aerobic conditions, and bacteria that do grow are selected for by the nutrients added to the medium. Thus R_2A selects for heterotrophic bacteria, while elemental sulfur selects for autotrophic sulfur oxidizers. Overall, it is estimated that only 0.1 to 1% of all soil bacteria can be cultured. Therefore, dilution and plating of soil bacteria only accounts for culturable bacteria and underestimates the true viable soil population by one to two orders of magnitude. An example of heterotrophic bacterial colonies that resulted from soil dilution and plating is shown in **Figure 1**. Note that approximately 1 million bacterial cells are needed for a colony to be visible to the naked eye.

This experiment demonstrates the dilution and spread plating methodology used to enumerate the number of bacteria within a soil sample. Specifically, two media are used: one designed for all bacteria, and the other that selects for actinomycetes. Once the bacterial colonies have grown on the agar plates, isolate the pure cultures of selected colonies by using a streak plate technique. Such pure cultures can then be further analyzed and characterized for specific traits and functions.

Procedure:

1. First Period:

    1.1. Preparation of Soil Dilutions for Plating.

        1.1.1. Prepare a dilution series of each of the soils (**Figure 2**).

            1.1.1.1. For each soil sample, suspend 10 g to a 95 ml water blank. Shake the suspension well.

            1.1.1.2. Before the soil settles in the bottle, remove 1 ml of the suspension with a sterile pipette and add it to a 9 ml water blank. Vortex thoroughly.

            1.1.1.3. Repeat the previous step three times, each time with a fresh 9 ml water blank and sterile pipette. This results in dilutions of ca. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} g soil ml^{-1} (tubes A through E).

    1.2. Making Spread Plates for Bacteria.

1.2.1. Prepare two or three spread plates for each dilution 10^{-3}, 10^{-4}, 10^{-5}, as follows. After vortexing, place a 0.1 ml drop of each dilution (this increases the effective dilution by a factor of ten) to three separate, labeled peptone-yeast agar plates. Inoculate no more than three plates before spreading, as standing allows too much liquid to be absorbed into the agar in one spot.

1.2.2. Take the glass hockey stick spreader, dip it in ethanol, and flame the spreader in a Bunsen burner just long enough to ignite the ethanol.

1.2.3. Move the spreader out of the flame and hold it above the first of the inoculated plates to allow all of the ethanol to burn off. Then quickly open the plate, holding the lid nearby in one hand. Touch the spreader to the agar away from the inoculum to cool it, and spread the drop of inoculum around on the surface of the agar until all traces of free liquid disappear (the surface becomes somewhat tacky).

1.2.4. Replace the lid, reflame the spreader, and repeat with the next plate. Work quickly so as not to contaminate the agar with airborne organisms.

1.2.5. Incubate the bacteria plates (inverted) at room temperature for 1 week.

1.3. Making Spread Plates for Actinomycetes.

1.3.1. Use the dilutions 10^{-2}, 10^{-3}, and 10^{-4} from the previous steps. Spread plate 0.1 ml of vortexed suspension on glycerol-casein pastes as before, making three replicates for each dilution.

1.3.2. Incubate the actinomycete plates (inverted) at room temperature for 2 weeks.

2. Second Period:

2.1. Counting Bacteria (after 1 week incubation).

2.1.1. Examine all of the bacteria plates carefully. Note the differences in colony size and shape. When grown on agar, bacteria produce slimy colonies ranging from colorless to brightly-colored orange, yellow, or pink colonies. In contrast, actinomycetes have a filamentous growth habit that makes it possible to visually distinguish them from the bacteria. Actinomycete colonies are chalky, firm, leathery, and break under pressure. In contrast, bacterial colonies smear under pressure.

2.1.2. Count the total number of bacterial colonies (CFUs) for each plate, including any actinomycetes. Average the totals for each dilution. Count only the plates with a countable dilution (30-200 colonies per plate).

2.1.3. Calculate the sample mean of CFUs per gram of dry soil for each of the soils.

2.2. Isolation of Pure Cultures.

2.2.1. Select 5 individual bacterial colonies from any of the plates. Use a high dilution plate, as it tends to have pure colonies that are separated well. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other. Include an actinomycete as one of the colonies.

2.2.2. Sterilize the loop by dipping it in alcohol and flaming it.

2.2.3. Quickly open the Petri dish of interest, and touch the loop to a bare spot in the agar to cool it. Then, remove a small amount of a colony of interest onto the loop.

2.2.4. Open a fresh peptone-yeast plate and quickly make a streak, as shown by streak A in **Figure 3**.

2.2.5. Sterilize the loop again, touch a bare spot on the agar on the new plate, and make a streak like streak B in **Figure 3**, crossing streak A only on the first pass. If streak A is crossed again, then isolating individual colonies won't be successful.

2.2.6. Repeat the previous step, making streak C in **Figure 3**, crossing only streak B on the first pass.

2.2.7. Finish by making streak D as in **Figure 3**, crossing only streak C on the first pass. If performed properly, this technique results in individual colonies growing on streak D or sooner, as the number of cells on the loop have been sufficiently diluted to individual cells.

3. Third Period:

3.1. Counting Actinomycetes (after 2 week incubation).

3.1.1. Examine all of the actinomycete plates carefully. Note differences in colony size and shape.

3.1.2. Count the total number of actinomycete colonies (CFUs) for each plate, subtracting any bacteria. Average the totals for each dilution. Count only those plates with a countable dilution (as with the bacteria).

3.1.3. Calculate the sample mean of actinomycete CFUs per gram of dry soil for each of the soils.

Results:

A 10-gram sample of soil with a moisture content of 20% on a dry weight basis is analyzed for viable culturable bacteria via dilution and plating techniques. The dilutions were made as shown in Table 1. 1 ml of solution E is pour plated onto an appropriate medium and results in 200 bacterial colonies.

$$\text{Number of CFU per g moist soil} = \frac{1}{\text{dilution factor}} \times \text{number of colonies}$$

$$= \frac{1}{10^5} \times 200 \text{ CFU/g moist soil}$$

$$= 2.00 \times 10^7 \text{ CFU/g moist soil}$$

But, for 10 g of moist soil,

$$\text{Moisture content} = \frac{\text{moist weight} - \text{dry weight (D)}}{\text{dry weight (D)}}$$

Therefore,

$$0.20 = \frac{10 - D}{D} \text{ and}$$

$$D = 8.33 \text{ g}$$

$$\text{Number of CFU per g dry soil} = 2.00 \times 10^7 \times \frac{1}{8.33} = 2.4 \times 10^7$$

Applications:

There are two fundamental applications of dilution and plating of soil bacteria. The first application is the enumeration of culturable bacteria within a particular soil. The quantification of the number of soil bacteria gives an indication of soil health. For example, if there are 10^6 to 10^8 culturable bacteria present per gram of soil, this would be considered a healthy number. A number less than 10^6 per gram indicates poorer soil health, which may be due to a lack of

nutrients as found in low organic matter soils; abiotic stress imposed by extreme soil pH values (pH < 5 or > 8); or toxicity imposed by organic or inorganic anthropogenic contaminants.

The second major application is the visualization and isolation of pure cultures of bacteria. The pure cultures can subsequently be characterized and evaluated for specific characteristics that may be useful in either medical or environmental applications. Examples include: antibiotic production; biodegradation of toxic organics; or specific rhizobia useful for nitrogen fixation by leguminous crops, such as peas or beans.

Legend:

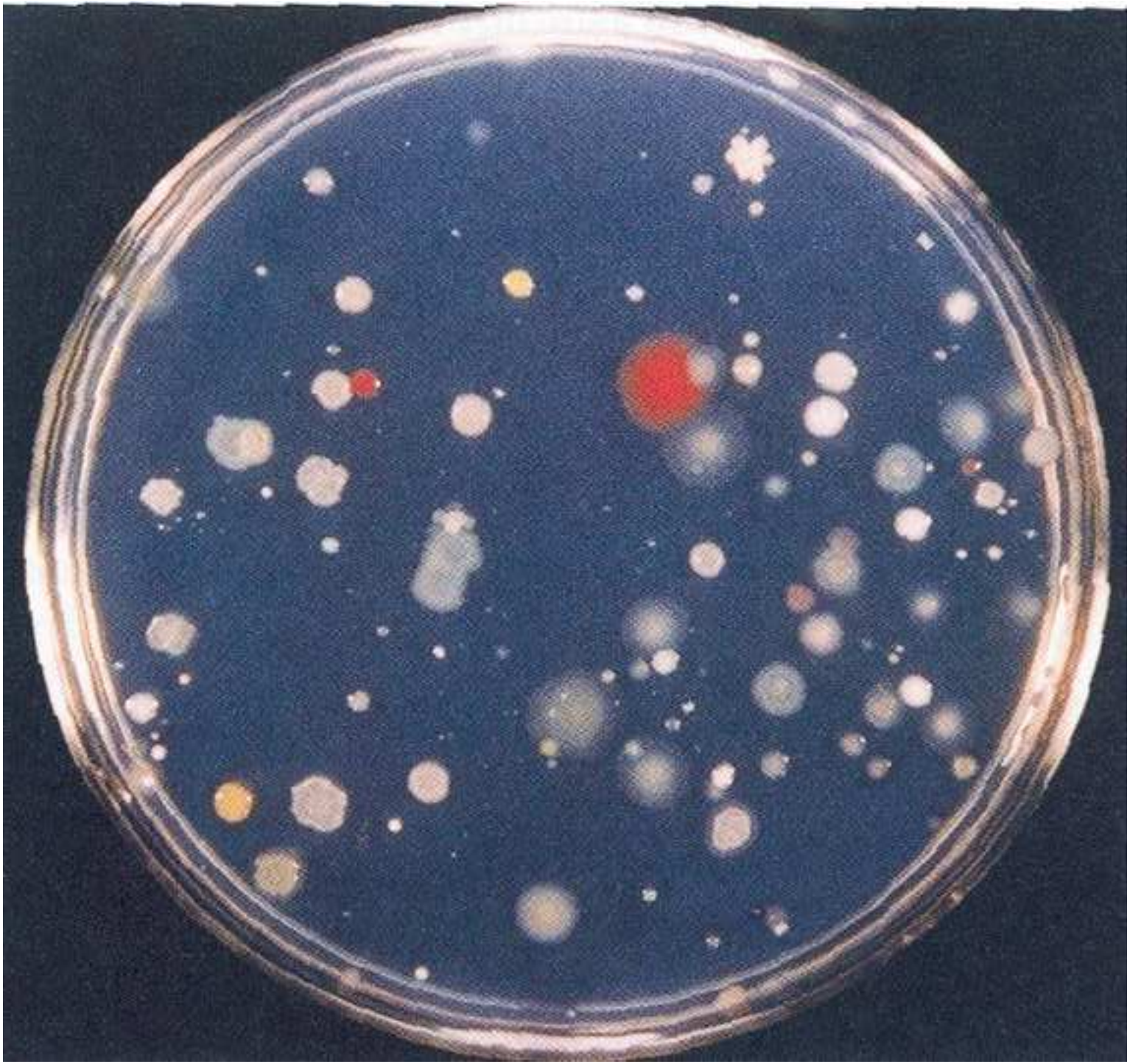
Figure 1: Heterotrophic colonies on an R_2A agar plate. A number of discrete colonies with diverse morphology arise after dilution and plating from soil. Permission for use granted by Academic Press.

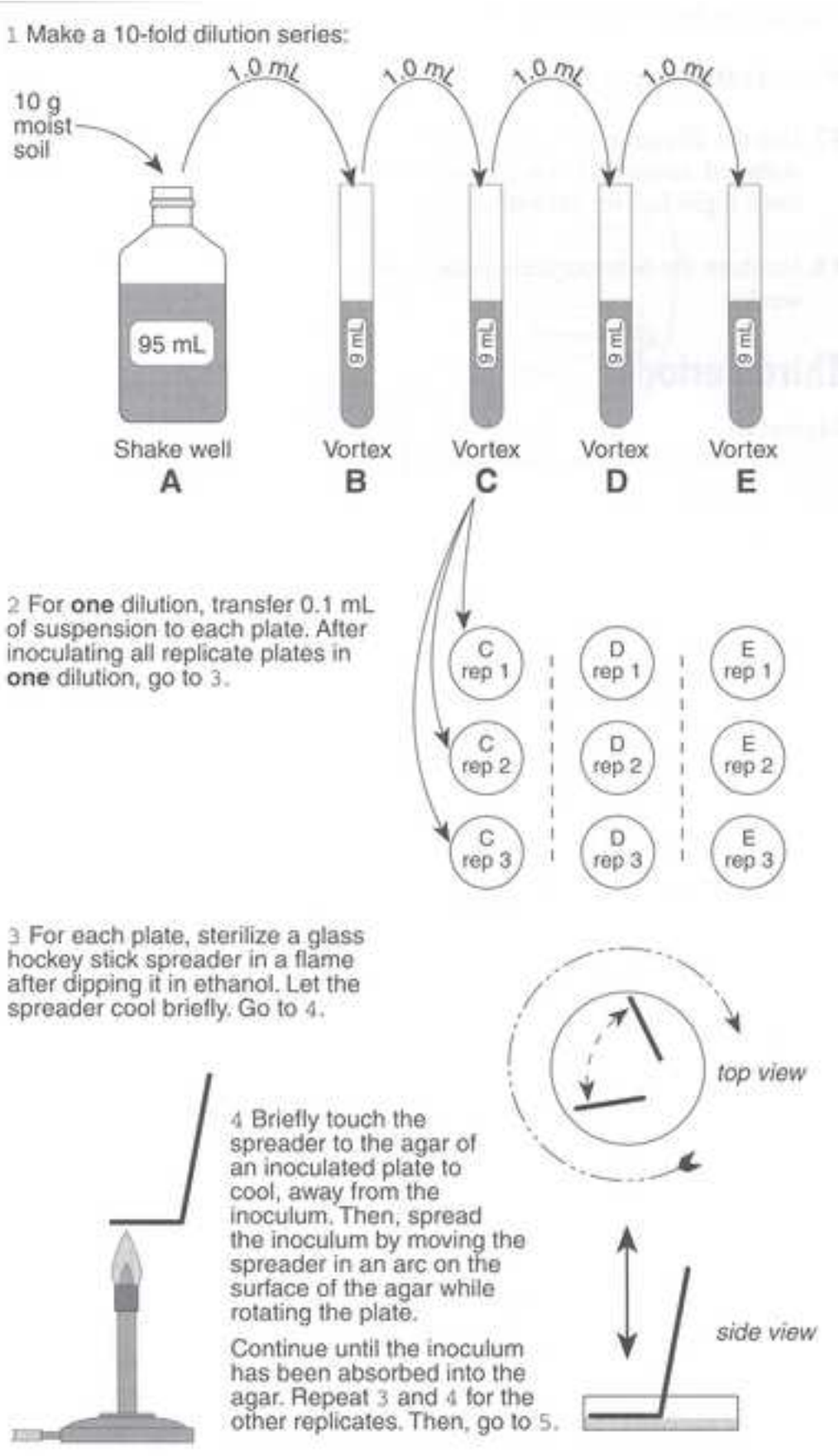
Figure 2: A schematic showing the procedure for viable heterotrophic plate counts of bacteria used in this experiment. Use tubes B, C, and D for the actinomycetes, likewise plating 0.10 ml of soil suspension. Permission for use granted by Academic Press.

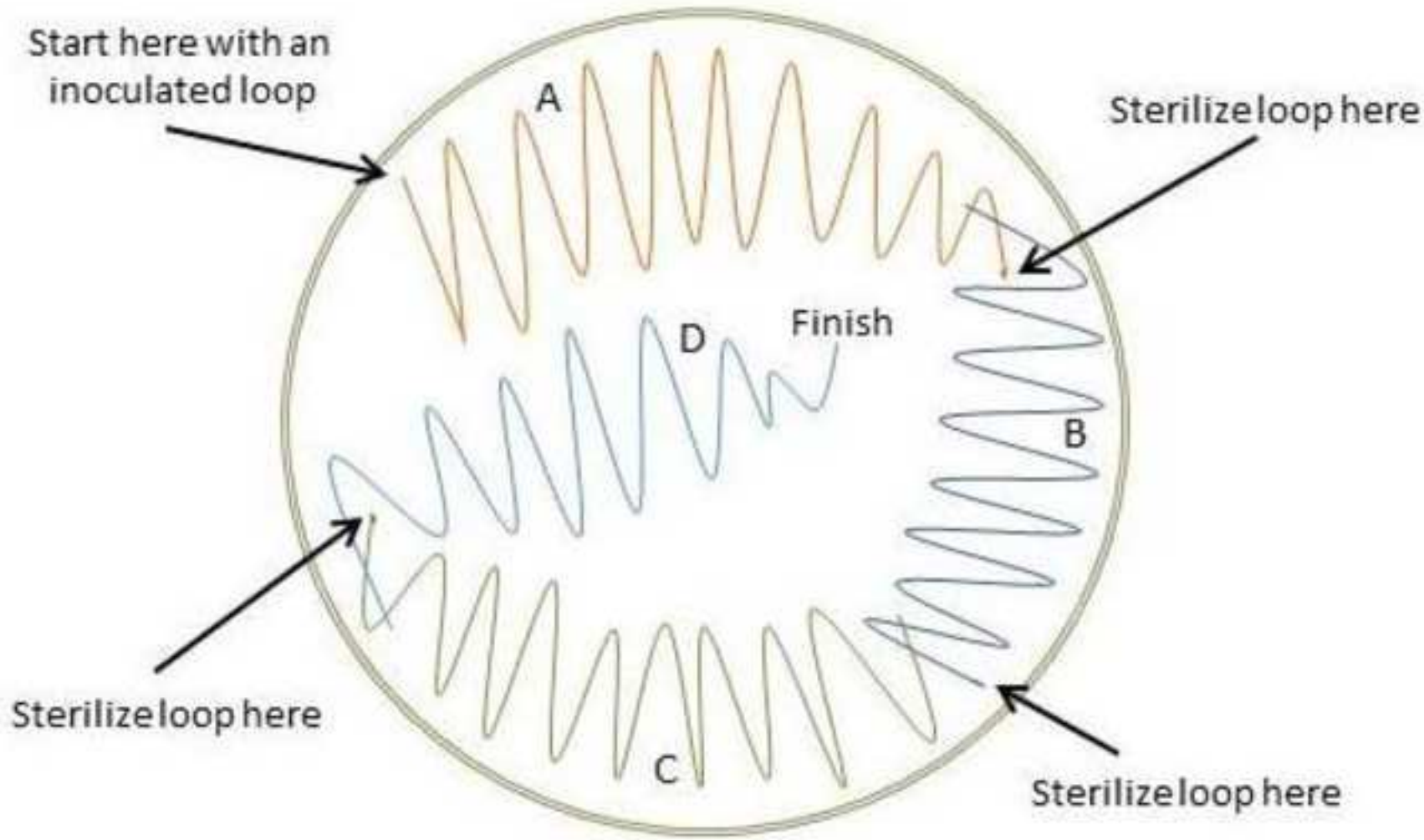
Figure 3: Isolation of pure cultures via streak plates.

Table 1: Dilution and plating of the samples.

Click here to download Photo or Graphic File: Ian Pepper_Visualizing Bacterial Colonies_Figure 1.jpg







Dilution and Plating Calculations

Step		Dilution
10 g soil (weight/volume)	95 ml saline (solution A)	10^{-1}
1 ml solution A (volume/volume)	9 ml saline (solution B)	10^{-2}
1 ml solution B (volume/volume)	9 ml saline (solution C)	10^{-3}
1 ml solution C (volume/volume)	9 ml saline (solution D)	10^{-4}
1 ml solution D (volume/volume)	9 ml saline (solution E)	10^{-5}

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Overview:

Surface soils are a heterogeneous mixture of inorganic and organic particles that combine together to form secondary aggregates. Within and between the aggregates are voids or pores that visually contain both air and water. These conditions create an ideal ecosystem for bacteria, so all soils contain vast populations of bacteria, usually up to 1,000 million per grams of soil.

Bacteria are the simplest of microorganisms, known as prokaryotes. Within this prokaryotic group, there are the filamentous microbes known as actinomycetes. Actinomycetes are actually bacteria, but they are frequently considered to be a unique group within the classification of bacteria because of their filamentous structure, which consists of multiple cells strung together to form hyphae~~, hey are frequently considered to be a unique group within the classification of bacteria~~. This experiment~~In this experiment we~~ uses glycerol case media that select for actinomycete colonies, during dilution and plating. Typically, actinomycetes are approximately 10% of the total bacterial population. Bacteria and actinomycetes are found in every environment on Earth, but the abundance and diversity of these microbes in soil is unparalleled. These microbes are also essential for human life and affect what people eat, drink, breathe, or touch. In addition, there are bacterial species that can infect people and cause disease, and there are bacteria that can produce natural products capable of healing people. ~~, including antibiotics such as streptomycin from actinomycetes.~~Actinomycetes are particularly important for producing antibiotics, such as streptomycin. Bacteria are critical for nutrient cycling, plant growth, and degradation of organic contaminants.

Bacteria are highly diverse in terms of the number of species that can be found in soil, in part because they are physiologically and metabolically diverse. Bacteria can be heterotrophic, meaning they utilize organic compounds, such as glucose, for food and energy, or autotrophic, meaning they utilize inorganic compounds, such as elemental sulfur, for food and energy. They can also be aerobic, utilizing oxygen for respiration, or anaerobic, utilizing combined forms of oxygen, such as nitrate or sulfate, to respire. Some bacteria can use oxygen or combined forms of oxygen and are known as facultative anaerobes.

One way to enumerate the number of bacteria present in a soil sample is to utilize dilution and plating methodology. This methodology utilizes agar as a medium for bacterial growth, a process termed, “culturable technology.” Because of the vast numbers of bacteria found within soils, a small sample of soil is serially diluted in water, prior to being plated on agar within a Petri plate. Typically, a small amount of soil contained within 0.1 to 1 ml of the diluted soil suspension is “spread” over the surface of the agar plate. The plates contain agar, which is molten when hot, but solid when cool. In addition to the agar, nutrients, such as peptone yeast

or a product commercially available as R_2A, are added to the medium to allow for the growth of heterotrophic bacteria.

Dilution and plating is an inexpensive and relatively simple technology for the enumeration of soil bacteria. However, there are several drawbacks to the technique. Some common errors and assumptions associated with dilution and plating assays are as follows: it is assumed that every single soil bacterium gives rise to a colony, but in reality a colony may arise from a clump of cells, resulting in an underestimation of true culturable count. During serial dilution of the soil, soil particles can settle out (fall to the bottom), so the true aliquot of soil is not passed on into the next dilution. Many soil microbes are viable but non-culturable. Slow growing bacteria may not result in visible colonies within a reasonable time frame (1-2 weeks).

Also, anaerobic bacteria do not grow under aerobic conditions, and bacteria that do grow are selected for by the nutrients added to the medium. Thus R_2A selects for heterotrophic bacteria, while elemental sulfur selects for autotrophic sulfur oxidizers. Overall, it is estimated that only 0.1 to 1% of all soil bacteria can be cultured. Therefore, dilution and plating of soil bacteria only accounts for culturable bacteria and underestimates the true viable soil population by one to two orders of magnitude. An example of heterotrophic bacterial colonies that resulted from soil dilution and plating is shown in **Figure 1**. Note that approximately 1 million bacterial cells are needed for a colony to be visible to the naked eye.

This experiment demonstrates the dilution and spread plating methodology used to enumerate the number of bacteria within a soil sample. Specifically, two media are used: one designed for all bacteria, and the other that selects for actinomycetes. Once the bacterial colonies have grown on the agar plates, isolate the pure cultures of selected colonies by using a streak plate technique. Such pure cultures can then be further analyzed and characterized for specific traits and functions.

Comment [AW1]: Why is this different? What makes this group unique?

Comment [ILP2]: See paragraph #2 on first page

Procedure:

~~**Materials**~~
~~Moist soil samples~~
~~Benchtop balance (±0.01 g)~~
~~9 peptone yeast agar plates per soil type~~
~~9 glycerol-casein agar plates amended with cycloeximide~~
~~1 sterile, 95 ml water blank for each soil type~~
~~4 sterile, 9 ml water blanks for each soil type~~
~~10 sterile, 1 ml pipettes for each soil type~~
~~Pipette bulb~~
~~1 test tube rack~~
~~Glass hockey stick spreader~~
~~Ethyl alcohol for flame sterilization~~
~~Vortex gas burner pre-prepared R_2A agar plates pre-prepared glycerol-casein agar plates~~

1. First Period:

1.1. Preparation of Soil Dilutions for Plating~~.~~

1.1.1. Prepare a dilution series of each of the soils (**Figure 2**).

1.1.1.1. For each soil sample, ~~suspend~~add 10 g of soil into a 95~~-~~ml water blank. Shake the suspension well.

1.1.1.2. Before the soil settles in the bottle, remove 1 ml of the suspension with a sterile pipette and add it to a 9~~-~~ml water blank. Vortex thoroughly.

1.1.1.3. Repeat the previous step three times, each time with a fresh 9 ml water blank and sterile pipette. Remember to agitate each tube prior to removing the 1 ml sample, so the~~at~~ soil does not settle. This results in dilutions of ca. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} g soil ml^{-1} (tubes A through E).

1.2. Making Spread Plates for Bacteria~~.~~

1.2.1. Prepare two or three spread plates for each dilution 10^{-3}, 10^{-4}, 10^{-5}, as follows. After vortexing, place a 0.1 ml drop of each dilution. ~~-~~The use of 0.1 ml ~~(this~~ increases the effective dilution by a factor of ten~~,~~) and this is taken into account in the calculation of the number of bacteria contained within the soil ~~-~~to three separate, labeled peptone-yeast agar plates. Inoculate no more than three plates before spreading, as standing allows too much liquid to be absorbed into the agar in one spot.

1.2.2. Take the glass hockey stick spreader, dip it in ethanol, and flame the spreader in a Bunsen burner just long enough to ignite the ethanol.

1.2.3. Move the spreader out of the flame and hold it above the first of the inoculated plates to allow all of the ethanol to burn off. Then quickly open the plate, holding the lid nearby in one hand. Touch the spreader to the agar away from the inoculum to cool it, and spread the drop of inoculum around on the surface of the agar until all traces of free liquid disappear (the surface becomes somewhat tacky).

1.2.4. Replace the lid, reflame the spreader, and repeat with the next plate. Work quickly so as not to contaminate the agar with airborne organisms.

1.2.5. Incubate the bacteria plates ~~(inverted)~~ at room temperature for 1 week. Make sure the plates~~Plates should be~~ are inverted during the incubation to prevent drops of moisture from condensation from falling onto the agar surface.

1.3. Making Spread Plates for Actinomycetes~~.~~

1.3.1. Use the dilutions 10^{-2}, 10^{-3}, and 10^{-4} from the previous steps. Spread plate 0.1 ml of vortexed suspension on glycerol-casein plat~~ast~~es as before, making three replicates for each dilution. The lower dilutions are used, since actinomycetes are typically one tenth of the total bacterial population.

1.3.2. Incubate the actinomycete plates (inverted) at room temperature for 2 weeks.

2. Second Period:

2.1. Counting Bacteria (after 1 week incubation)~~.~~

2.1.1. Examine all of the bacteria plates carefully. Note the differences in colony size and shape. When grown on agar, bacteria produce slimy colonies ranging from colorless to brightly-colored orange, yellow, or pink colonies. In contrast, actinomycetes have a filamentous growth habit that makes it possible to visually distinguish them from the bacteria. Actinomycete colonies are chalky, firm, leathery, and break under pressure. In contrast, bacterial colonies smear under pressure. Thus, touching any colony with a sterilized loop allows for actinomycete colonies to be distinguished.

2.1.2. Count the total number of bacterial colonies (CFUs) for each plate, including any actinomycetes. Average the totals for each dilution. Count only the plates with a countable dilution (30-200 colonies per plate).

2.1.3. Calculate the sample mean of CFUs per gram of dry soil for each of the soils.

2.2. Isolation of Pure Cultures~~.~~

2.2.1. Select at least 5 individual bacterial colonies from any of the plates. More colonies can be selected if there is particular interest in the soil. Use a high dilution plate, as it tends to have pure colonies that are separated well. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other. Include an actinomycete as one of the colonies.

2.2.2. Sterilize the loop by dipping it in alcohol and flaming it.

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2.2.6. Repeat the previous step, making streak C in **Figure 3**, crossing only streak B on the first pass.

2.2.7. Finish by making streak D as in **Figure 3**, crossing only streak C on the first pass. If performed properly, this technique results in individual colonies growing on streak D or sooner, as the number of cells on the loop have been sufficiently diluted to individual cells.

3. Third Period:

3.1. Counting Actinomycetes (after 2 week incubation)~~.~~

3.1.1. Examine all of the actinomycete plates carefully. Note differences in colony size and shape.

3.1.2. Count the total number of actinomycete colonies (CFUs) for each plate, subtracting any bacteria. Average the totals for each dilution. Count only those plates with a countable dilution (as with the bacteria).

3.1.3. Calculate the sample mean of actinomycete CFUs per gram of dry soil for each of the soils.

Results and Calculation:

A 10-gram sample of soil with a moisture content of 20% on a dry weight basis is analyzed for viable culturable bacteria via dilution and plating techniques. The dilutions were made as shown in **Table 1**. 1 ml of solution E is pour~~-~~plated onto an appropriate medium and results in 200 bacterial colonies.

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$$= 2.00 \times 10^7 \text{ CFU/g moist soil}$$

But, for 10 g of moist soil,

$$\text{Moisture content} = \frac{\text{moist weight} - \text{dry weight (D)}}{\text{dry weight (D)}}$$

Therefore,

$0.20 = \frac{10 - D}{D}$ and

$D = 8.33$ g

$$\text{Number of CFU per g dry soil} = 2.00 \times 10^7 \times \frac{1}{8.33} = 2.4 \times 10^7$$

Applications:

There are two fundamental applications of dilution and plating of soil bacteria. The first application is the enumeration of culturable bacteria within a particular soil. The quantification of the number of soil bacteria gives an indication of soil health. For example, if there are 10^6 to 10^8 culturable bacteria present per gram of soil, this would be considered a healthy number. A number less than 10^6 per gram indicates poorer soil health, which may be due to a lack of nutrients as found in low organic matter soils; abiotic stress imposed by extreme soil pH values (pH < 5 or > 8); or toxicity imposed by organic or inorganic anthropogenic contaminants.

The second major application is the visualization and isolation of pure cultures of bacteria. The pure cultures can subsequently be characterized and evaluated for specific characteristics that may be useful in either medical or environmental applications. Examples include: antibiotic production; biodegradation of toxic organics; or specific rhizobia useful for nitrogen fixation by leguminous crops, such as peas or beans.

Comment [AW3]: These would be good applications to show. Which can you demonstrate in lab Preferably 2.

Comment [ILP4]: It is very difficult to demonstrate microscopic bacteria. We could get images of a root system with root nodules.

Legend:

Figure 1: Heterotrophic colonies on an R_2A agar plate. A number of discrete colonies with diverse morphology arise after dilution and plating from soil. Permission for use granted by Academic Press.

Figure 2: A schematic showing the procedure for viable heterotrophic plate counts of bacteria used in this experiment. Use tubes B, C, and D for the actinomycetes, likewise plating 0.10 ml of soil suspension. Permission for use granted by Academic Press.

Figure 3: Isolation of pure cultures via streak plates.

Table 1: Dilution and plating of the samples.