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# Environmental Microbial Ecology

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**Abstract:** In this exercise, students develop and test a hypothesis about microbial ecology. The hypothesis that students choose must be testable with the "EcoPlate," a multiwell test plate that allows rapid determination of the metabolic diversity of a bacterial population without tedious and time-consuming reagent preparation by the instructor. After inoculation with a solution of bacteria washed from a soil or plant sample, the plate returns a unique set of positive (purple) and negative (clear) reactions that allows students to assess whether or not two samples contain similar or dissimilar bacterial populations.

## Introduction

In this open-ended inquiry exercise, students develop and test a hypothesis about microbial ecology. Specifically, the hypothesis that students choose must be testable with the "EcoPlate," a multi-well test plate that allows rapid determination of the metabolic diversity of a bacterial population without tedious and time-consuming reagent preparation by the instructor. After inoculation with a solution of bacteria washed from a soil or plant sample, the plate returns a unique set of positive (purple) and negative (clear) reactions that allows students to assess whether or not two samples contain similar or dissimilar bacterial populations. It can also, given certain assumptions, allow students to evaluate whether one sample contains a more physiologically diverse assemblage of bacteria than another sample. This type of analysis is termed the "community level physiological profiling," or CLPP, of natural microbial diversity. This exercise was developed for use in a sophomore-level ecology and evolution class at the University of Pittsburgh at Bradford. For this course, the Principal Investigator (PI) System was used and students worked in groups of three to prepare a proposal, complete their experiment and submit a lab report. Students anecdotally responded that they enjoyed developing their own experiment with the EcoPlates, and the PI groups playfully competed with each other for the one whose project resulted in the fastest color changes. Students participating in the lab exercise appeared to develop a new appreciation for microbes and their physiology, a greater respect for sterile techniques, and a healthy and realistic degree of frustration with the fact that even small experiments require considerable forethought, and that the data generated are frequently hard to interpret.

# Student Outline

## Introduction

Microbes, including bacteria, form an important part of all ecosystems. A few of the many processes impacted by bacteria are the decomposition and replenishment of nutrients in our soils, the production of usable nitrogen for some of our important crops, the spread of disease in agricultural, human, and natural populations of plants and animals, and the digestive process in many mammals. Bacteria have also proven to be incredibly useful in the field of biotechnology. For example, human genes have been inserted into bacteria to produce human growth hormone to treat people with the genetic disease of dwarfism.

In 1990, a Norwegian research group used new molecular biology techniques to estimate the bacterial diversity in soil, and found that there could be as many as 4000 different species (or genomes) of bacteria in a single gram of soil! More recent theoretical models have estimated that pristine soils uncontaminated with pollutants contain up to $8.3 \times 10^5$ bacterial species per gram of soil (Gans *et al*., 2005). This diversity is truly astounding and, in commentary, scientists have compared the number of bacterial species in soil to the numbers of stars in our galaxy (Curtis and Sloan, 2005). This incredible diversity of bacteria remains a wide-open field for ecological research. In addition to recent advances in DNA analysis, low-prep commercially available technologies have been developed to help characterize bacteria in natural environments. These methods are based on the fact that the environment for each bacterium can contain variable sets of organic compounds and each strain or species of bacteria tends to have the capacity to select from unique sets of organic compounds as their food sources. Thus, a mixture of bacteria will likewise have the capacity to metabolize a limited and unique set of carbon compounds. Analyses in which bacterial populations are characterized by the types of carbon sources they can use are called "community level physiological profiling," or CLPP.

In today's lab, we will use of "EcoPlates," 96-well cell culture plates that contain 31 important carbon sources (substrates) repeated three times along with controls. The carbon sources are listed at the end of this handout (Fig. 1), and nine of these substrates are plant root exudates (Campbell *et al*., 1997). We will inoculate these plates with samples that contain bacteria from natural settings and allow them to incubate over time. A tetrazolium dye has also been added to each well. If there are any bacteria in the sample that use a particular carbon source, formazan will be produced and the well will turn purple (Preston-Mafham *et al*., 2002). In this lab, we will quantify "functional diversity" which is very different from species diversity. Each species of bacteria can use multiple carbon sources (for example, *Bacillus thuringiensis* can typically use 13 of the 31 carbon sources.) Thus the microbial diversity value that we will obtain has a physiological definition: i.e., how diverse is the population in terms of its ability to feed on various carbon compounds, rather than how diverse is the population in terms of the number of different species.

What other types of microbial analyses exist and how do they compare to the EcoPlate reactions?

*Identifying Bacteria by Microscopic Examination*

Bacteria can be examined under microscopes, and a few bacteria have distinctive shapes or colonies of cells. They are typically only several microns in length. Bacterial shape and aggregation patterns are rarely sufficient to identify bacterial species or to assess bacterial diversity.

*Identifying Bacteria with Culturing and Chemical Tests*

Historically, bacteria were cultured in the lab on agar media, and then a series of biochemical tests in the laboratory could be used to identify the isolated bacteria. For example, Gram's test was developed by the Danish pharmacist, Hans Christian Joachim Gram. He discovered that some bacteria were blue after staining with crystal violet dye while others remain colorless. Gram initially developed this method without an understanding of how the two types of bacteria differed, but we now know that Gram positive (blue) and Gram negative (colorless) bacteria differ in the composition and structure of their cell walls. Specifically, Gram negative bacteria have less peptidoglycan in their cell membranes. Today many other tests can be used to compare unknown bacterial types. One disadvantage of the agar culturing technique is that many bacterial species will not grow on this medium.

*Identifying Bacteria with DNA techniques*

Increasingly, bacteria are being identified using DNA-based techniques. These techniques often utilize the polymerase chain reaction (PCR-) and amplify small amounts of DNA extracted from bacteria millions of times. DNA techniques tend to be more expensive and more complicated, but they reveal the presence of the great number of bacteria that can otherwise not be cultivated on agar plates.

*Assessing Bacterial Diversity with Biolog plates*

Among other products, Biolog, Inc. sells EcoPlates, GramNegative Plates and GramPositive Plates. The latter two plates can help identify bacterial species in clinical settings. The EcoPlate is designed just to look at bacterial assemblages without specifically identifying particular bacterial species. The advantage is that these plates are ready-made, easy to use and generate useful information about microbial populations in a relatively short period of time. However, the plates are still essentially a culturing technique, and bacteria that will not grow in the wells will not be detected. Furthermore, there are many issues surrounding the interpretation of Biolog plates, depending on how long the plates are incubated, how concentrated the original bacterial suspensions were, and the conditions under which the plates are incubated. These issues can strongly affect the interpretation of EcoPlate data.

*Summary*

Many techniques exist for examination of mixed bacterial populations. Kirk *et al*. (2004) reviewed these techniques, and students are encouraged to look over the review.

**Methods**

*Part I Methods: Developing your question & proposal*

Each group has been provided with 6 EcoPlates. You are being asked to develop a question about bacterial communities found in different natural environments using these plates. The EcoPlate plates will give you a "metabolic fingerprint" of a particular community and the question that you choose to ask will probably be one of the following three types:

1. Spatial comparison: "Is the bacterial community found here the same as the one found there?"
2. Temporal comparison: "Is the bacterial community the same now as it will be later?"
3. Manipulative experiment: "Does the bacterial community change when something is added to or taken away from its habitat?" or "Does the bacterial community change when this environmental condition is altered?"

Your experiment may be quantitative and take samples at six levels of a known factor (and therefore use regression or correlation analysis) or your experiment may be qualitative and compare two or three discrete groups.

Once you have chosen a question to pursue, each group must develop a hypothesis to test with their six EcoPlates. As an example, we are interested in liverworts (a small plant related to mosses) and asked the following basic question: Do two samples of a single epiphytic liverwort species collected from over a mile apart harbor the same bacterial flora? One hypothesis would be that that each plant species has a unique and characteristic set of secondary compounds and so a single plant species should have the same bacterial flora associated with them. If the pattern of color change on the EcoPlates had been identical for all inoculated plates, then this hypothesis would be supported. If the EcoPlates gave a different pattern, this hypothesis would have been rejected.

The EcoPlates gave different patterns for samples of the same species from different localities, and the preliminary conclusion from the experiment is that there is not a consistent flora of bacteria contained within this particular epiphytic liverwort species. Many possible follow-up questions (and appropriate experiments) can be examined. For example, is there a smaller distance scale for which consistency among samples of the same plant species might be found? Are the communities of bacteria in the epiphytic plants the same as those found on the associated bark substrate? Would a different conclusion be drawn for a different plant species?

As you develop your hypothesis and question, please keep in mind that a "site" for bacteria can be anything from the leaf of a particular plant to the soil in a particular area. In no way should you feel limited to the study of soil bacteria, and you are encouraged to consider questions regarding the interactions between bacteria and other species (plants and animals). You are expected to turn in a proposal in the same format as used for other PI laboratory exercises in this course.

*Part II Methods: Sampling Bacteria from Natural Environments*

Since you have been provided with 6 EcoPlates, you will be taking 6 samples. You must devise a method for sampling the soil, plant matter, or other material in such a way that the hypothesis you have proposed will be adequately tested. Will you sample haphazardly? Will you sample randomly? How will you choose your sites? How often will you sample the site? The answers to these questions depend entirely on what hypothesis you have decided to pursue.

*Part III Methods: Inoculating your EcoPlate*

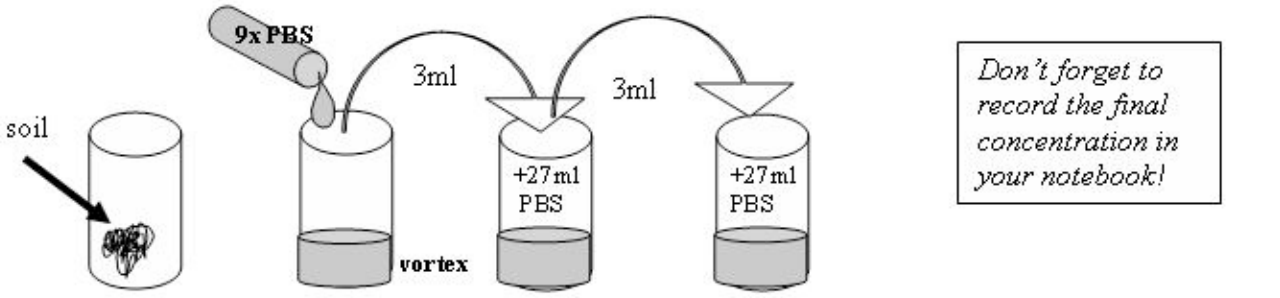
The first step in inoculating your plates will be to wash the bacteria from your sample by mixing your sample in a sterile buffer. You will next dilute that sample to a slightly cloudy suspension. Finally, you will inoculate the EcoPlate using the multichannel pipettor and incubate it for about four days at room temperature.

1. Collect your sample. It is essential that you do not contaminate your sample with bacteria from your hands or other places. Sterile test tubes with lids are provided, and if at all possible, you should collect your samples directly into these sterile tubes without any utensils. To avoid contamination by unwanted bacteria here are a few tips:
   Do not touch the soil or plant that you plan to sample with your hands or fingers.
   Do not choose samples from soil or plants that you think someone has touched.

Do not breathe or sneeze on your samples or into your tubes.
Do not leave your sterile tubes uncapped and open for airborne bacteria to enter.
Continue to use sterile techniques when diluting your sample in step #2.

If you will need to take your samples with forceps or other tools in order to get the sample into the sterile tubes, please talk to your instructor about a suitable disinfection procedure for your tools. Several grams per sample should be more than enough for the next step.

2. After you have taken your samples to the lab, you will need to dilute them in sterile "phosphate buffered saline" or PBS provided by your instructor. Mix your sample in sterile PBS in a set dilution (wt/vol) so that you dislodge the bacteria into the solution. To get good results from your EcoPlate you don't want too many or too few bacteria, just the "right" number. You will have to guess to a certain extent at your dilution, depending on what is being sampled. In our experience, you want to dilute the sample until it is barely turbid (barely cloudy). This is often a $10^3$ dilution for soil (1 g of soil/1000 ml of PBS buffer), but could be $10^2$ for plants (1 g of plant matter/100 ml of PBS buffer). Figure 1 provides a pictorial description of the dilution procedure and should be examined before beginning your dilution.




BE CAUTIOUS NOT TO CONTAMINATE YOUR VIALS DURING THIS PROCESS. DO NOT BREATH INTO THEM AND CAP THEM AS SOON AS POSSIBLE.

**Figure 1.** Instructions for the serial dilution of your soil or plant sample.

3. For each vial/flask of buffer diluted sample, pour the 50 ml into either the bottom half or the top half of a sterile Petri dish. Using the multichannel pipettor, add 100 ul of the plate wash suspension to inoculate each well in the EcoPlate. In order to learn the technique, the entire class will watch a demonstration of the inoculation of a plate. Try to avoid touching the sides or bottom of the wells with the pipette tips. If possible, hold the tips just above the well to release the liquid and do not rest the pipett tips on any part of the EcoPlate.

4. Place your plates in a plastic bag and incubate them at room temperature. A small piece of moistened paper towel inside the bag will help to maintain a constant high level of humidity and avoid evaporation from the wells. You will need to check your plate daily and fill out a data sheet showing which wells give a positive reaction (purple color) daily. It usually takes about 4 days for most of the positive wells to change color. Students should be cautious to keep the plates horizontal (flat) and not tip the plates while examining them today or subsequent days.

**Data Collection**

For experiments that are not examining a microbial community over time, you should inoculate all your EcoPlates in the same lab period and then examine them every day until the color begins to change. Record the "metabolic fingerprint" for each plate on the first day of color change and for three subsequent days. A sample data sheet is provided at the end of this handout that you can photocopy. You should mark down which wells were purple and which wells were not purple on the datasheet.

After recording the data, go back and look through the data sheets. Was there a day on which the plates appeared to stop changing color and, if so, were there some positive and some negative wells on those days or were all the wells positive? Use only the data from the particular day on which the color changes appeared to have ceased. For example, if day 4 showed the fewest *additional* purple wells, then you should use the data for day 4 for ALL your plates. If all your wells ultimately showed positive purple results, speak to your instructor.

**Question to Consider**

All technology has its limitations, and EcoPlates are no exception. As you analyze your results please consider the following questions:

1. How might temperature affect your results? Do all bacteria grow equally well at room temperature? How are we influencing the results by incubating the EcoPlate at room temperature?
2. How might the pH of the PBS buffer affect your results? Do you think you might get a different fingerprint for a community depending on the pH of the PBS buffer you use? Do you think you might get different results for a single pure bacterial species depending on the pH of the buffer used?
3. Could antagonistic interactions between bacteria in your sample affect the pattern on EcoPlates?
4. What if protists exist in your sample; could they affect the EcoPlate patterns? What about fungi?
5. If you accidentally touched the wells during pipetting into the first 8 wells and then continued to pipette into the remaining wells with the same tips, how might this affect your results?
6. How could you test whether your PBS buffer was sterile?
7. What is wrong with assuming that the number of purple wells on the EcoPlate corresponds to species diversity?

8. For microbes, do you think that species diversity or physiological functional diversity is more important ecologically? Defend your answer.
9. If your sample includes bacterial species A, B, and C, but there are many more of bacterial species A, how would this affect the results? How might the pattern on the EcoPlate differ from a sample that contained A, B & C in equal amounts?
10. Could the sample suspension that you inoculated have additional metabolizable organic material (e.g. dissolved sugars) in it? If your bacterial suspension contained dissolved sugars, how might this affect your results?
11. What happens to the bacterial population inside a well after a well is inoculated? Do things stay the same, do all components of the population increase at the same rate, and can all organic compounds be metabolized easily or are some more labile and others more recalcitrant?

**Data Analysis**

Data analysis will depend on the question you asked. However, here are some suggestions on how to analyze your data:

- You can calculate a single diversity index per sample called % functional diversity = 100 X (number of positive carbon source wells)/31
    - This value varies from 0 to100% with 0 being low diversity and 100% being highly diverse.

  *For this lab, functional diversity refers to microbial nutritional diversity and capabilities rather than species diversity!*

- You can compare two samples by calculating the following measures of community similarity:

  Simple Matching Coefficient ($S_{SM}$) = (a + d)/(a+b+c+d), where:

  a = Number of carbon sources used by both sample A and sample B
  b = Number of carbon sources used by Sample B but not by Sample A
  c = Number of carbon sources used by Sample A but not by Sample B
  d = Number of carbon sources not used by bacteria in either sample

  - You should consider whether to include the carbon sources that show inconsistent positive reactions. You should indicate in the lab report how you scored the carbon sources that displayed inconsistent results among the three replicates.
  - If the two samples give identical "fingerprints" on the EcoPlates, $S_{sm}$ will be 1.0. If the two samples give exactly opposite "fingerprints", the value will be 0.

- You will need to report the inconsistency found within each sample:

  % Variation of Results within Sample = 100 X (i/31), where:

  i = the number of carbon sources in which the three replicates were not ALL positive or ALL negative.

**List of EcoPlate Carbon Sources**

2-Hydroxy Benzoic Acid
4-Hydroxy Benzoic Acid
D,L-α-Glycerol Phosphate
D-Cellobiose
D-Galactonic Acid γ-Lactone
D-Galacturonic Acid
D-Glucosaminic Acid
D-Malic Acid
D-Mannitol
D-Xylose
Glucose-1-Phosphate
Glycogen
Glycyl-L Glutamic Acid
i-Erythritol
Itaconic Acid
L-Arginine
L-Asparagine
L-Phenylalanine
L-Serine
L-Threonine
N-Acetyl-D-Glucosamine
Phenylethylamine
Putrescine
Pyruvic Acid Methyl Ester
Tween 40
Tween 80
α-Cyclodextrin
α-D-Lactose
α-Ketobutyric Acid
β-Methyl-D-Glucoside
γ-Hydroxybutyric Acid

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 Water | A2 β-Methyl-D-Glucoside | A3 D-Galactonic Acid γ-Lactone | A4 L-Arginine | A1 Water | A2 β-Methyl-D-Glucoside | A3 D-Galactonic Acid γ-Lactone | A4 L-Arginine | A1 Water | A2 β-Methyl-D-Glucoside | A3 D-Galactonic Acid γ-Lactone | A4 L-Arginine |
| B1 Pyruvic Acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparagine | B1 Pyruvic Acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparagine | B1 Pyruvic Acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparagine |
| C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L-Phenylalanine | C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L-Phenylalanine | C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L-Phenylalanine |
| D1 Tween 80 | D2 D-Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine | D1 Tween 80 | D2 D-Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine | D1 Tween 80 | D2 D-Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine |
| E1 α-Cyclodextrin | E2 N-Acetyl-D-Glucosamine | E3 γ-Hydroxybutyric Acid | E4 L-Threonine | E1 α-Cyclodextrin | E2 N-Acetyl-D-Glucosamine | E3 γ-Hydroxybutyric Acid | E4 L-Threonine | E1 α-Cyclodextrin | E2 N-Acetyl-D-Glucosamine | E3 γ-Hydroxybutyric Acid | E4 L-Threonine |
| F1 Glycogen | F2 D-Glucosaminic Acid | F3 Itaconic Acid | F4 Glycyl-L-Glutamic Acid | F1 Glycogen | F2 D-Glucosaminic Acid | F3 Itaconic Acid | F4 Glycyl-L-Glutamic Acid | F1 Glycogen | F2 D-Glucosaminic Acid | F3 Itaconic Acid | F4 Glycyl-L-Glutamic Acid |
| G1 D-Cellobiose | G2 Glucose-1-Phosphate | G3 α-Ketobutyric Acid | G4 Phenylethyl-amine | G1 D-Cellobiose | G2 Glucose-1-Phosphate | G3 α-Ketobutyric Acid | G4 Phenylethyl-amine | G1 D-Cellobiose | G2 Glucose-1-Phosphate | G3 α-Ketobutyric Acid | G4 Phenylethyl-amine |
| H1 α-D-Lactose | H2 D,L-α-Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine | H1 α-D-Lactose | H2 D,L-α-Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine | H1 α-D-Lactose | H2 D,L-α-Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine |

FIGURE 1. Carbon Sources in EcoPlate

**Figure 2** taken from Biolog literature and showing the distribution of the carbon sources throughout the 96-well plate.

## Notes for the Instructor

We have found it helpful to have the students complete a worksheet to practice the data analysis the week before they begin the lab. This worksheet is provided in Appendix C.

Although the math itself is simple, the analysis can be quite tedious. An excel sheet has been created by the authors whereby the pattern of color change on the EcoPlate can be typed into a grid on an excel sheet (1 for purple, 0 for clear). The excel sheet then provides all the comparisons and calculations listed in the materials section. Instructors should decide whether they want to provide the students with that excel sheet or whether they would rather the students analyze by hand, and instructors use the excel sheet to check the students work. The excel sheet can be obtained by emailing Mary Mulcahy (mnp1@pitt.edu).

Most students find it hard to remember to use sterile techniques, and you have to watch them closely so that they do not contaminate their experiment. For example, if not supervised carefully, students will use graduated cylinders or scoopulas off the shelf instead of using the sterile tubes that you provide. Students will frequently let the tips of the pipette touch the wells of the EcoPlate or place the lids of sterile tubes face down on an unsterile surface.

Although this experiment is set up as an open-ended inquiry exercise, the experiment could easily be altered to be a structured whole-class experiment. This would probably make the experiment a little cheaper. A whole group experiment on, for example, the role of fertilization on soil microbial diversity would make an interesting structured ecology lab. Likewise, we found that salted soil showed dramatically different bacterial communities than unsalted soil.

For at least one of our orders of the EcoPlates, they were in such demand that it took three weeks to receive all 50 of them. The plates arrived sealed individually in packages with a two-year expiration date. For example, when ordered in October of 2005 and the expiration date was for 2007. In other words, it probably is worth it to order these well in advance and then simply store them until you need them, although they do take up some space. They must be stored in the refrigerator until use.

Enough were ordered for each group to use 6 plates. In retrospect, 7 plates would be more useful since that would permit every group to have a control plate inoculated with supposedly sterile buffer.

Additional useful references are: Biolog (2005), Niklinska *et al.* (2004), Merkeley *et al.* (2005), Preston-Mafham *et al.* (2002), and Kirk *et al.* (2004).

## Materials and Equipment

1. Multichannel 8-well pipette
   - Single Pipettors or even eyedroppers can be used, but students will take considerably longer filling their plates.
   - Do not use Scienceware/Bel-Art - Transpette™ 8 Channel Transfer Pipettor
   - These are disposable multichannel pipettes, and it is our experience that this will not work well.

2. Sterile pipette tips if you are using a non-disposable multichannel pipette. Each group project will need at least 6 sterilized tips per EcoPlate, but mistakes happen, and it is wiser to have 12 tips per plate. Ideally, have a sterilized box of pipette tips all full and ready to use for each group project.

3. Sterile Centrifuge Tubes (05-526B, FisherSci) 50-ml with graduations marked or sterilized lidded glassware. These are going to be vortexed so should be size appropriate for vortexing. Each group project will require about 13 sterilized tubes/vials but extras may be handy. Six of these tubes/vials will be used to collect the soil or leaves. A small portion (0.05 g for soil & 0.10 g for plants) of the collected material will be transferred to new sterile tubes. One additional tube is useful for pouring the buffer into the tubes before they are vortexed.

4. Biolog EcoPlates (approximately $10/ plate)
   Toll Free Orders : 1-800-284-4949
   Telephone : (510) 785-2564
   Fax : (510) 782-4639
   http://www.biolog.com/contactUs.html

5. Sterile PBS solution. Using the methods described here, each student group will need at least 50 ml of buffer per EcoPlate inoculated, and therefore, each group will need 300 ml per project involving 6 EcoPlates. For 5 students groups: two liters of the solution will provide enough with some to spare. This solution can be made in a 2 L volumetric flask. Next, 200 ml of the liquid can be transferred to each of ten 500 ml Erlenmeyer flasks covered loosely with aluminum foil (only 200 ml per flask is recommended since the flask should be less than half full before autoclaving). The ten flasks can be autoclaved (we used 45 minutes on liquid cycle to 121 degrees C). After autoclaving, the aluminum foil can be tightened, the solution should be cooled, and preferably the students will use the solution that day or the following day. Again, schools without autoclaves can still do this lab. They can use whatever sterilization techniques are available to them, and then run a control plate in which they inoculate with the "supposedly" sterile buffer alone.

   Recipe for PBS:
   8.5 gm NaCl
   0.3 gm $KH_2PO_4$
   1.12 gm $Na_2HPO_4.7H_2O$
   Filled to 1L with distilled $H_2O$
   pH 6.8
   This solution should be sterilized before use (autoclave or filter-sterilization is recommended).

6. Sterile Petri dishes for transferring buffer to EcoPlate. Each group project will require one half of a Petri dish per EcoPlate, and therefore 6 halves for 6 EcoPlates. Students can be provided with disposable sterile dishes or, even better, freshly autoclaved glass Petri dishes. One possibility is to autoclave the glass Petri dishes in sets of 6 halves on loose sheets of aluminum foil and then wrap the 6 halves tightly in the sterilized aluminum foil. Each student group can then be given the sterile wrapped dishes ready for use.

7. Sterile scoopulas or spatulas. As above, stainless steel utensils (even from a used-thrift store) can be autoclaved on loose sheets of aluminum foil and then wrap 6 utensils in the sterilized aluminum foil for each student group.

8. Electronic scales with to weigh to at least two decimal places (0.01 g)

9. Ziplock bags (large or small are fine; more are needed if small are purchased); these are to put the EcoPlates into after inoculation so that they stay humid and don't dry out entirely.

10. (Optional) Sterilization devices for students (ethanol and Bunsen burners, for example.)

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## About the Authors

**Mary Puterbaugh Mulcahy** has a Ph.D. from the University of Missouri at Columbia. Among other courses, she teaches ecology and evolution at the University of Pittsburgh at Bradford where she is an associate professor of biology. Her research interests are in plant-animal interactions and plant mating systems. She is currently studying the relationship between a dioecious leafy liverwort species and the rotifers that live within its lobular leaves.

**Hank Edenborn** is an environmental microbiologist with a Ph.D. from Rutgers University. He works at the U.S. Department of Energy's National Energy Technology Laboratory in Pittsburgh, PA. His current research interests include the microbial ecology and bioremediation of historically industrially-contaminated soils and sediments; the role of lichens, bryophytes, and higher plants as habitats for bacteria; and the microbial diversity of unusual natural environments found within the state of Pennsylvania.

# Appendix A: Data Sheet

Student Name _____
Description of Sample: _____
_____
_____
Date Sample was Collected: _____
Date EcoPlate was Inoculated:_____
Date of Data Collection*:_____
* Students should collect data four times: on the first day that any purple wells are observed and the following four days. Students may skip data collection on Saturday and Sunday if a weekend interrupts this time period.
Location Collected: _____
Dilution (wt/volume buffer): _____
Other Comments: _____
_____
_____

In the figure below, write P for clearly purple wells, L for light purple wells, and U for wells that you are not certain whether they are positive (purple) or not. All clear wells can be left blank.

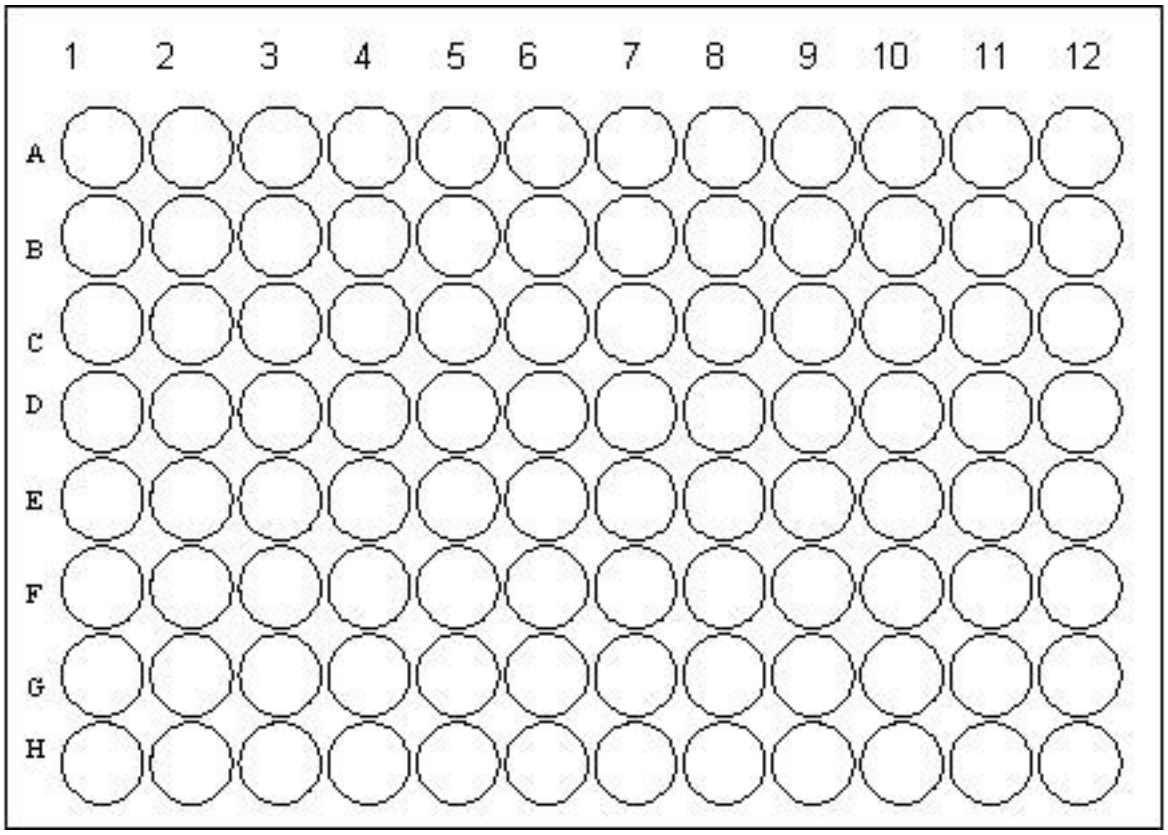


**Figure 3.** A blank EcoPlate that can be used for data collection.

# Appendix B: Sample Project, Data and Analysis

Example of a Student Project:

In the Allegheny National Forest near Bradford, PA, drilling for oil is a common occurrence. One student group wanted to explore some of the environmental consequences of this drilling. Drilling for oil in our area also involves pumping up salt or brine water in addition to the oil. Today, this brine water must be transported and disposed of safely. However, 50 years ago, this was not the case, and the salt water was simply dumped around the pump site. One student group wanted to ask how the brine water affected soil bacteria. They chose to do a controlled experiment rather than search for an actual well with the brine pollution.

The students took the 50 ml sterile tubes to a relatively undisturbed natural area on campus. The student scooped soil into the tubes avoiding touching the soil with anything other than the sterile tubes. The student randomly chose which tubes to receive the salt treatment and which ones would be controls. The students put 5 ml of Instant Ocean (artificial seawater) into the three "treatment" tubes and 5 ml of distilled water into the three "control" tubes. As a measure of control over the sterility of the graduated cylinder and distilled water, the students put 30 ml of distilled water into the supposedly sterile graduated cylinder and then removed 5 ml for each control tube. The remaining 15 ml were mixed with the appropriate weight of "Instant Ocean" and 5 ml of the instant ocean was added to each of the treatment tubes. They repeated this for three days. The following week, they took 6 more sterile tubes and diluted the soil with the buffer. They chose to put just the cap of the sterile tube on the scale, tare the scale and then use a sterilized spatula to remove just enough soil to bring the weight to approximately 0.05 g. The tube was filled with approximately 50 ml of sterile PBS and the cap was put on the tube. The tube was vortexed for 1 minute.

The solution from the tube was then poured into half of a sterilized glass Petri dish. The multichannel pipette was used to put 100 ul of solution into each well on the EcoPlate. This was repeated for all 6 tubes of soil. Here are the data collected six days after inoculation of the EcoPlates, where wells marked 1 were purple or light purple and those marked 0 were clear:

**Untreated Sample 1**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| B | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| C | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| D | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| E | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| F | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| G | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| H | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |

Total carbon sources that were consistently positive for all three replicates: 7
Total carbon sources that were consistently negative for all three replicates: 7
Total carbon sources positive for 2 of the 3 replicates: 6
Total carbon sources positive for 1 of the 3 replicates: 11
% Functional Diversity: 24/31 = 0.774
** We chose to regard the carbon source as being used if ANY of the three replicate wells for that carbon source were used.

**Untreated Sample 2**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| B | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| C | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| E | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| F | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| G | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| H | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |

Total carbon sources that were consistently positive for all three replicates: 5
Total carbon sources that were consistently negative for all three replicates: 16
Total carbon sources positive for 2 of the 3 replicates: 8
Total carbon sources positive for 1 of the 3 replicates: 2
% Functional Diversity: 15/31 = 0.484

**Untreated Sample 3**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| B | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| C | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| E | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| F | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| G | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| H | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |

Total carbon sources that were consistently positive for all three replicates: 13
Total carbon sources that were consistently negative for all three replicates: 7
Total carbon sources positive for 2 of the 3 replicates: 4
Total carbon sources positive for 1 of the 3 replicates: 7
% Functional Diversity: 24/31 = 0.774

**Instant Ocean Treated Sample #1**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| B | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| D | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| F | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| G | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Total carbon sources that were consistently positive for all three replicates: 1
Total carbon sources that were consistently negative for all three replicates: 25
Total carbon sources positive for 2 of the 3 replicates: 1
Total carbon sources positive for 1 of the 3 replicates: 3
% Functional Diversity: 6/31 = 0.194

**Instant Ocean Treated Sample #2**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| B | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| D | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| G | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Total carbon sources that were consistently positive for all three replicates: 1
Total carbon sources that were consistently negative for all three replicates: 27
Total carbon sources positive for 2 of the 3 replicates: 2
Total carbon sources positive for 1 of the 3 replicates: 1
% Functional Diversity: 4/31 = 0.129

**Instant Ocean Treated Sample #3**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| B | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| C | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| D | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| F | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| G | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Total carbon sources that were consistently positive for all three replicates: 0
Total carbon sources that were consistently negative for all three replicates: 16
Total carbon sources positive for 2 of the 3 replicates: 3
Total carbon sources positive for 1 of the 3 replicates: 12
% Functional Diversity: 15/31=0.484

Anecdotal comment: ALL of the positive wells on the treated plates were light colored whereas many of the wells on the control (untreated) plates were dark purple. This experiment showed that the addition of salt water had two effects on the soil: 1) it reduced diversity of the bacteria and 2) it reduced the overall abundance of the bacteria. From this evidence it can be tentatively concluded that disposal of brine associated with oil drilling would have a negative effect on the microbial diversity of the surrounding soil.

# Appendix C: Homework for Students to Complete Before Beginning Lab

How do you tell one bacterial species from another? How can you tell whether a person is sick with *Streptococcus pneumoniae* or *Staphylococcus aureus*? Most of us are familiar with identifying vertebrate species and plants based on morphological features. However, bacteria vary relatively little for morphological features. Bacteria species can be lumped into groups based on the shape of the cells (rod-shaped, coccoid, chains of cells, etc.), sometimes color, and sometimes colony appearance (is the edge of the bacterial colony wavy or smooth, for example), but these physical features are rarely sufficient to fully classify bacterial to species.

Despite the lack of morphologically variable features, bacterial species certainly do vary from one another; rather than morphological features, the wealth of features that distinguish bacteria are biochemical. Some biochemical features that distinguish bacteria are contained within the structure of the cells themselves. In the 1800's, a Danish research Hans Christian Gram discovered that crystal violet will stain some bacterial strains but not others. Today, Gram's stain continues to be one of the most commonly used methods of separating bacteria. Gram-positive bacteria tend to have more peptidoglycan than Gram-negative, and stain more darkly and more permanently than the Gram-negative bacteria. Many human pathogens are Gram-negative bacteria, and their ability to cause disease appears to be related to their cell walls and to the presence of endotoxins there.

Bacterial species also differ in what they are able to eat, which is another biochemical source of variation since it is biochemical enzymes that determine what types of compounds bacteria can digest. Substances that bacteria can eat or can be grown on are sometimes called "substrates". Today, we will use a test plate produced by Biolog, Inc. to study variation in biochemistry (specifically metabolic capabilities) within and among different bacterial communities. Every community of bacteria has a characteristic set of carbon sources that can be used by the bacteria within that community and has a characteristics set of carbon sources which are not usable by any member of that community. That pattern of what substrates or carbon sources can and can not be used can be considered a metabolic "fingerprint". We will be comparing the fingerprints of different communities, and you will have the task of developing a specific question to ask.

The EcoPlates that we use today are intended for microbial ecologists, but the same company (Biolog, Inc.) makes very similar test plates called "Gram-Negative Plates" and "Gram-Positive Plates," that can be read by a spectrophotometer and have more clinical applications. These GN and GP-plate systems may actually make it into the clinical medical setting and are already being used in research.

*Community-Level Physiological Profiling (CLPP)*

Each species of bacteria has a specific set of carbon compounds that can and can not be used for energy. In fact, this set of compounds is usually unique at the species level with a limited amount of variability (some mutants of particular species will "lose" the ability to use a carbon source, but rarely will a mutant "gain" the ability to use a brand new carbon source that is not used by other wild-type members of that species). Likewise, each community of bacteria (all the bacterial species that live in one particular area) also have a specific set of other carbon sources that can and can not be used for energy. Describing that pattern of carbon-usage is called "community-level physiological profiling (CLPP)".

Next week, your group will be provided with 6 EcoPlates. Your group is asked to develop a question about bacterial communities found in different natural environments using these EcoPlates. Your experiment may be quantitative and take samples at six levels of a known factor (and therefore use regression or correlation analysis) or your experiment may be qualitative and compare two or three discrete groups. Before you begin developing your question, please practice scoring the EcoPlates.

*Learning to Score An EcoPlate*

Figure 4 below shows the layout of carbon substrates on our EcoPlates. Each substrate (carbon source) has been given a unique number. Referring to Table 2, you can see which substrate (carbon source) is found in each of the wells. For example, substrate #10 is i-Erythritol. Like all the substrates here, i-Erythritol is found three times on the EcoPlate. It is found in the following wells: C2, C6, and C10.

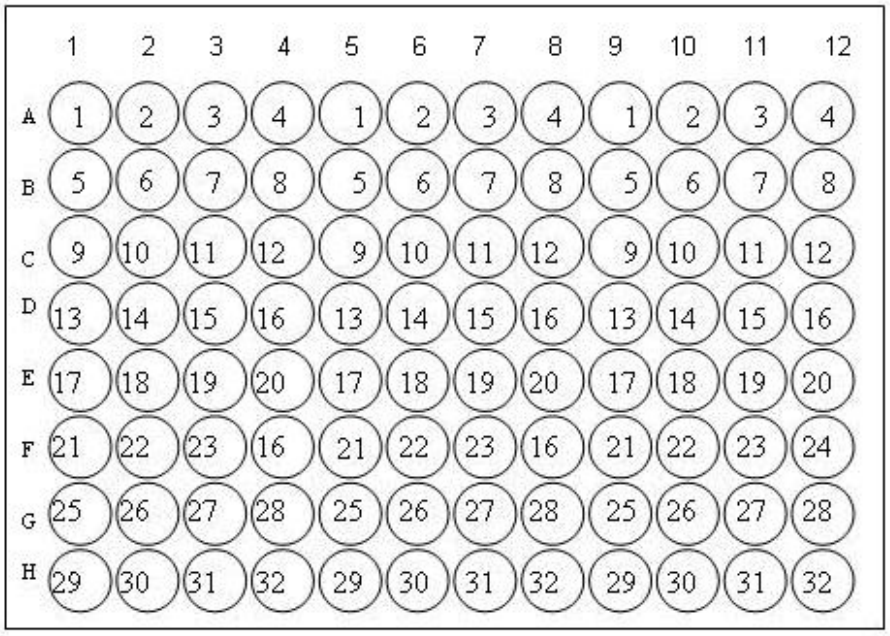


**Figure 4.** A drawing of an EcoPlate in which the circles are wells. A single substrate and a dye are present in each well. The substrate is a unique carbon source that may or may not be used as energy by the bacteria in your sample. The numbers inside the circles each correspond to a particular substrate. Please refer to Table 1 to see what substrate corresponds to what number.

Prior to beginning your experiment, please practice scoring the EcoPlates. There are three main calculations that you should be able to do *and* to understand:

$$\% \text{ Functional diversity} = 100 * \frac{\text{number of positive (purple/pink) carbon source wells}}{\text{Total number of carbon source wells (31)}}$$

This value varies from 0 to100% with 0 being low diversity and 100% being highly diverse.

You will need to report the inconsistency found within each sample.

$$\% \text{ Variation of Results within Sample} = 100 * i/31$$

i = the number of carbon sources in which the three replicates were not ALL positive or ALL negative. On Table 1 (on a subsequent page), anywhere there is a 1 or 2 the plate returned an inconsistent result and should be counted.

$$\% \text{ Similarity } (S_{SM}) = 100 * \frac{a + d}{a + b + c + d}$$

*Please note that water is not a carbon source.

a = Number of carbon sources used by both sample A and sample B
b = Number of carbon sources used by Sample B but not by Sample A
c = Number of carbon sources used by Sample A but not by Sample B
d = Number of carbon sources not used by bacteria in either sample

If the two samples give identical "fingerprints" on the EcoPlates, $S_{sm}$ will be 100. If the two samples give exactly opposite "fingerprints", the value will be 0. If a plate has an inconsistent result (a 1 or 2 in table 1), assume the carbon source is used and that the inconsistency was a result of low concentration of bacteria.

*Please score EcoPlates 1, 2, & 3 below by filling in table 1. The shaded wells are the ones that turned pink or purple.*

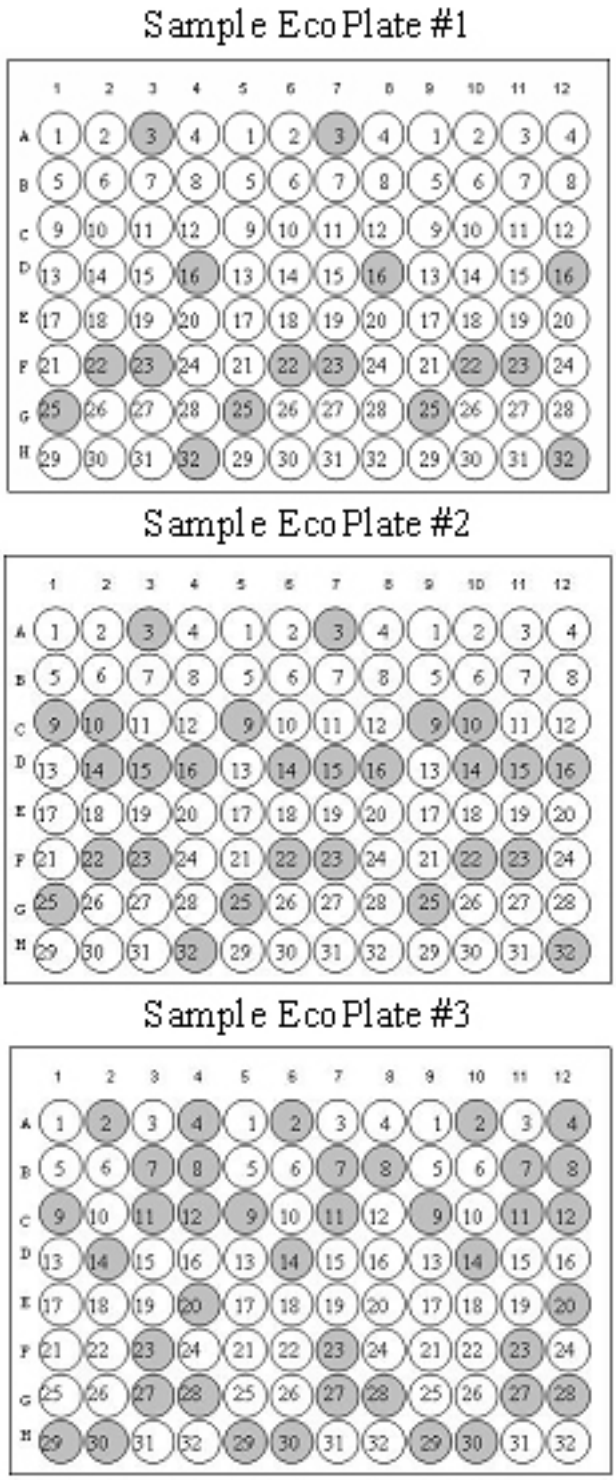


**Figure 5.** Invented data for student homework assignment.

**Table 1.** Use Figure 5 to fill in this table. Sample Plate #1 is already scored for you as an example. You need to score sample plates 2 and 3. Place a zero in the cell table if none of the three replicates turned pink or purple. Place a 1 in the table if ONE of the three replicates turned pink or purple. Place a 2 in the table if two of the three replicates turn pink or purple (one remains clear), and place a 3 in if all three replicates are positive (pink or purple).

| Substrate Name | Substrate # | Sample Plate #1 | Sample Plate #2 | Sample Plate #3 |
|---|---|---|---|---|
| Water | 1 | 0 | | |
| β-Methyl-D-Glucoside | 2 | 0 | | |
| D-Galactonic Acid γ-Lactone | 3 | 2 | | |
| L-Arginine | 4 | 0 | | |
| Pyruvic Acid Methyl Ester | 5 | 0 | | |
| D-Xylose | 6 | 0 | | |
| D-Galacturonic Acid | 7 | 0 | | |
| L-Asparagine | 8 | 0 | | |
| Tween 40 | 9 | 0 | | |
| i-Erythritol | 10 | 0 | | |
| 2-Hydroxy Benzoic Acid | 11 | 0 | | |
| L-Phenylalanine | 12 | 0 | | |
| Tween 80 | 13 | 0 | | |
| D-Mannitol | 14 | 0 | | |
| 4-Hydroxy Benzoic Acid | 15 | 0 | | |
| L-Serine | 16 | 3 | | |
| α-Cyclodextrin | 17 | 0 | | |
| N-Acetyl-D-Glucosamine | 18 | 0 | | |
| γ-Hydroxybutyric Acid | 19 | 0 | | |
| L-Threonine | 20 | 0 | | |
| Glycogen | 21 | 0 | | |
| D-Glucosaminic Acid | 22 | 3 | | |
| Itaconic Acid | 23 | 3 | | |
| Glycyl-L Glutamic Acid | 24 | 0 | | |
| D-Cellobiose | 25 | 3 | | |
| Glucose-1-Phosphate | 26 | 0 | | |
| α-Ketobutyric Acid | 27 | 0 | | |
| Phenylethylamine | 28 | 0 | | |
| α-D-Lactose | 29 | 0 | | |
| D,L-α-Glycerol Phosphate | 30 | 0 | | |
| D-Malic Acid | 31 | 0 | | |
| Putrescine | 32 | 2 | | |
| Total # of substrates used | | 6 | | |
| % Functional Diversity | | 19.35% | | |
| % Variation of Results within Sample | | 6.45% | | |

*Please Answer the Following Questions:*

(1 pt) Question 1: How many *different* carbon substrates are there on the EcoPlate? _____

(1 pt) Question 2: What is the name of the carbon substrate designated number 15? _____ (1 pt) Question 3: Using the letter to designate the row and numbers to designate column, list the wells containing substrate #15. (For example, i-Erythritol is found in C2, C6, and C10.)_____

(1 pt) Question 4: What is the control substrate in this plate? _____Should the control wells turn pink? _____

(1 pt) Question 5: Using the letter to designate the row and numbers to designate column, list the wells containing the control. _____

(1 pt) **Table 2.** Please fill in the information in this table based on your calculations

| Sample Plate | Functional Diversity | % Variation of Results |
|---|---|---|
| 2 | | |
| 3 | | |

(1 pt) **Table 3.** Please fill in the information on this table based on your calculations.

| | Percent Similarity | | |
|---|---|---|---|
| | Sample Plate 1 | Sample Plate 2 | Sample Plate 3 |
| Sample Plate 1 | | | |
| Sample Plate 2 | | | |
| Sample Plate 3 | | | 100 |

(1 pt) Question 6: Please use Table 3 to answer this question. These three plates came from the following sources:

Bacterial wash from the unwashed left hand of Joe Smith
Bacterial wash from the unwashed right hand of Joe Smith
Bacterial wash from the unwashed left hand of Jane Doe.

Based on Table 3, propose a hypothesis for which plate contains the bacterial community of Jane Doe's hand. Please explain your reasoning.____

_____

_____

_____

_____

# Appendix D: Key to Student Homework

**Table 1.**

| Substrate Name | Substrate # | Sample Plate #1 | Sample Plate #2 | Sample Plate #3 |
|---|---|---|---|---|
| Water | 1 | 0 | 0 | 0 |
| β-Methyl-D-Glucoside | 2 | 0 | 0 | 3 |
| D-Galactonic Acid γ-Lactone | 3 | 2 | 2 | 0 |
| L-Arginine | 4 | 0 | 0 | 2 |
| Pyruvic Acid Methyl Ester | 5 | 0 | 0 | 0 |
| D-Xylose | 6 | 0 | 0 | 0 |
| D-Galacturonic Acid | 7 | 0 | 0 | 3 |
| L-Asparagine | 8 | 0 | 0 | 3 |
| Tween 40 | 9 | 0 | 3 | 3 |
| i-Erythritol | 10 | 0 | 2 | 0 |
| 2-Hydroxy Benzoic Acid | 11 | 0 | 0 | 3 |
| L-Phenylalanine | 12 | 0 | 0 | 2 |
| Tween 80 | 13 | 0 | 0 | 0 |
| D-Mannitol | 14 | 0 | 3 | 3 |
| 4-Hydroxy Benzoic Acid | 15 | 0 | 3 | 0 |
| L-Serine | 16 | 3 | 3 | 0 |
| α-Cyclodextrin | 17 | 0 | 0 | 0 |
| N-Acetyl-D-Glucosamine | 18 | 0 | 0 | 0 |
| γ-Hydroxybutyric Acid | 19 | 0 | 0 | 0 |
| L-Threonine | 20 | 0 | 0 | 2 |
| Glycogen | 21 | 0 | 0 | 0 |
| D-Glucosaminic Acid | 22 | 3 | 3 | 0 |
| Itaconic Acid | 23 | 3 | 3 | 3 |
| Glycyl-L Glutamic Acid | 24 | 0 | 0 | 0 |
| D-Cellobiose | 25 | 3 | 3 | 0 |
| Glucose-1-Phosphate | 26 | 0 | 0 | 0 |
| α-Ketobutyric Acid | 27 | 0 | 0 | 3 |
| Phenylethylamine | 28 | 0 | 0 | 3 |
| α-D-Lactose | 29 | 0 | 0 | 3 |
| D,L-α-Glycerol Phosphate | 30 | 0 | 0 | 3 |
| D-Malic Acid | 31 | 0 | 0 | 0 |
| Putrescine | 32 | 2 | 2 | 0 |
| Total # of substrates used | | 6 | 10 | 14 |
| % Functional Diversity | | 19.35% | 32.26% | 45.16% |
| % Variation of Results within Sample | | 6.45% | 9.68% | 9.68% |

(1 pt) Question 1: How many *different* carbon substrates are there on the EcoPlate? __31____

(1 pt) Question 2: What is the name of the carbon substrate designated number 15? _4-Hydroxy Benzoic Acid_

(1 pt) Question 3: Using the letter to designate the row and numbers to designate column, list the wells containing substrate #15. (For example, i-Erythritol is found in C2, C6, and C10.) D3, D7, D11_

(1 pt) Question 4: What is the control substrate in this plate? __water___ Should the control wells turn pink? ___No (organisms can not use water for energy & the tetrazolium dye will only change color if the bacteria can metabolize the substrate.)_____

(1 pt) Question 5: Using the letter to designate the row and numbers to designate column, list the wells containing the control. __A1, A5, A9__

(1 pt) **Table 2.**

| Sample Plate | Functional Diversity | % Variation of Results |
|---|---|---|
| 2 | 32.26% | 9.68% |
| 3 | 45.16% | 9.68% |

(1 pt) **Table 3.**

| | Percent Similarity | | |
|---|---|---|---|
| | Sample Plate 1 | Sample Plate 2 | Sample Plate 3 |
| Sample Plate 1 | 100 | | |
| Sample Plate 2 | 87% | 100 | |
| Sample Plate 3 | 42% | 42% | 100 |

1 compared to 2: a=6,b=4,c=0,d=21; 2 compared to 3: a=3,b=11, c=7,d=10; 1 to 3: a=1,b=13,c=5,d=12

(1 pt) Question 6: Please use Table 3 to answer this question. These three plates came from the following sources:
Bacterial wash from the unwashed left hand of Joe Smith
Bacterial wash from the unwashed right hand of Joe Smith
Bacterial wash from the unwashed left hand of Jane Doe.

Based on Table 3, propose a hypothesis for which plate contains the bacterial community of Jane Doe's hand. Please explain your reasoning.____Based on the % similarity calculation, sample plates 1 and 2 are the most similar (the bacterial community is 87% similar between 1 &2). We would expect that Joe would have similar bacteria on both his hands and so Sample Plates 1 and 2 are probably both from Joe Smith. Jane Doe would be the wash contained in sample plate #3.

# Appendix E: Overview of the Principal Investigator (PI) System

A major element to learning and doing ecology is being able to design and analyze data from thoughtful experiments. At the University of Pittsburgh at Bradford, some of our courses have followed the lead of Bates College in Maine which is well known for organizing their laboratory courses in the Principal Investigator (PI) format. At Pitt-Bradford, the sophomore ecology and evolution laboratory is organized around several investigative labs in which students participate in choosing the question, the design, the analysis and write up of the report. For each of these labs, students work in groups of three students, just as a professional science project might involve collaboration among several scientists. Within each group and for each lab, one person is assigned to be the "Principal Investigator" or "PI." The group submits a proposal articulating the experiment design for their project. This design is graded (all three students receive the same grade, worth the same percentage of their total grade), and feedback is provided on the proposal. After feedback is received, students may begin their study, and all three students are expected to participate actively in gathering the data. The PI for the experiment turns in a single lab report with all three students as authors with the first author as the PI. The PI is expected to do the writing of the report. The lab report is written as a scientific research paper, and at Pitt-Bradford, only the PI receives the grade for that lab report. By the end of the course, all three students have each been a PI on one laboratory project and have been collaborators on two laboratory projects. Each PI laboratory project is devoted two lab sessions, one session for developing the question and introducing students to the materials and equipment available to them, and the other session for beginning their projects. However, most students end up using more than two weeks to complete their data collection. To provide some structure and consistency, students are given a broad topic within which to work. In the example shown here, the broad topic is environmental microbial ecology (and students are expected to use the EcoPlates) but otherwise, students are given nearly complete free range as to what specific questions they would like to ask. In the semester that this project was used, the other two broad topics were "Factors Affecting Species Diversity" (methods recommended were the use of either tree diversity or leaf litter invertebrate diversity, and all projects had to use Simpson's Index and Shannon's Index) and "Ecology of Species Interactions" (students were encouraged develop questions around goldenrod gall interactions or around competitive interactions of plants in a greenhouse setting). Students were allowed to use a single experimental design to complete two PI projects, where, for example, one PI headed up collecting data on leaf litter invertebrate diversity while the other PI used the same experiment to collect data on microbial diversity with the EcoPlates.