Using Microbial Ecology to Teach Experimental Design and Sampling Methods

Resource Type: Curriculum: Classroom

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Abstract

These three activities have students work in groups to address a quantitative approach to experimental design and sampling. Adequate consideration of these issues is necessary to ensure microbial ecology experiments represent microbiology in "the real world." In the first exercise students sample a simple community (composed of classroom materials), evaluate how much sampling is necessary, and quantify diversity using Simpson's index. The second activity is a simulated sampling exercise emphasizing spatial organization of microbes in the environment and its relationship to sampling methods. The third exercise addresses experimental design with an emphasis on designing correctly replicated experiments.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

Introduction.

Topics related to the ecology of microorganisms are of increasing interest in the undergraduate curriculum. This module, a set of three exercises, is designed to provide a resource for a lab or classroom activity that addresses microbial ecology at the level of sampling strategy and experimental design. These exercises will be relevant in courses where microbial ecology is addressed (e.g., general biology, microbiology, ecology) or where microbial ecology examples can be used to teach experimental design related to ecology. A wide variety of accessible ecosystems may be utilized if the instructor wishes to move on to apply the design in a laboratory or field exercise.

This activity may also be helpful for instructors in preparing students for doing independent research in other areas of biology. By doing these exercises, students will gain an appreciation for many fundamental principles of sound experimental design, measurement, and analysis and will avoid some common pitfalls in the planning and execution of more serious (and costly) projects.

Learning Objectives.

1. Identify challenges that microbial ecologists face in measuring and sampling for microbial presence and activity in the natural environment (Introduction)

2. Differentiate between species diversity and community structure as applied to microbial ecology (Activity 1)

3. Learn a method for quantifying diversity in a microbial community (Activity 1)

4. Design an appropriate sampling scheme that takes into account community structure and organization (temporal and spatial) as well as scaling issues (Activity 2)

5. Recognize how to build replication into experimental design and avoid some common pitfalls when sampling and measuring microbial systems (Activity 3)

Background.

The background knowledge students should have to complete this activity is embedded within it and additional preparation of the students is not necessary. Some major considerations when studying microorganisms in the environment are: how
do we detect them, how do we identify them, and how do we quantify their presence. The following considerations are addressed in the three exercises that comprise this activity.

1. **Detection and visualization of microscopic organisms.** Because direct visualization of microbes in a representational manner is difficult, this challenge emphasizes the importance of a very sound sampling strategy when obtaining microbes from the natural environment and making claims about their roles and numbers.

2. **Definition and differentiation of microbial taxa.** Microbes are not easily classified into species or even genera. Molecular methods of identification are helpful but can also introduce a different sort of sampling and analytical bias.

3. **Cultivation issues and ecological relevance.** Are the microbes captured and cultivated truly those that are most important in the ecosystem? How can we cultivate and study organisms whose needs we cannot identify?

4. **Interdependence.** Microbial interactions are often complex and can be extremely difficult to sort out. For example, consortia are pairs or groups of species interacting in such a tightly coupled manner that separation is nearly impossible due to metabolic interdependence.

5. **Adherence to surfaces.** Microbes most typically grow on surfaces—attached to mucosa, rock, plastic, wood, dust and soil particles, etc. Even in open water, the majority of microbes are surface attached on microscopic particles of organic or inorganic matter that provide energy and other nutritional compounds. This adherent state must be taken into account when designing sampling and measurement strategies.

The exercises are organized to address these issues in the following manner:

**Exercise 1. Measuring diversity**

When sampling an unknown microbial community, the researcher first must determine the level of variation and heterogeneity in the target area. Some initial sampling and analysis will help in answering the question: how many samples must be taken in order to best describe the diversity of the community? In this exercise, students do simulated sampling with simple materials in the classroom and construct a graph that quantitatively helps them make an informed decision about the community and sampling effort required. They quantify the diversity of their communities using the Simpson's index of diversity, a measure commonly used in ecology.

**Exercise 2. Community structure and organization**

In the heterogeneous microbial world, cells organize and interact at both small scale and higher levels based upon factors that influence them and the resources available. In this exercise, which compliments and extends Exercise 1, students will do a simulated sampling and analysis that emphasizes the spatial organization of microbes in the environment and how these considerations inform sampling approach.

**Exercise 3. Common pitfalls in the study of microbial communities—pseudoreplication**

After making a decision about how to sample and how many samples are needed, a next step is to make sure that experiments are replicated. A major pitfall in the design of experiments is to fall into the trap of doing an "easy" replication, which may not be a true independent replicate at all. In this exercise, students are challenged with several experimental questions and design setups and learn to distinguish true independent replicates from pseudoreplicates that don't contribute to the validity of experimental results.

**PROCEDURE**

**Materials.**

If each group does not have access to a computer, a calculator is desirable for computing Simpson's index of diversity, if this will be done in class. Students will also need scrap paper and/or paper to record notes. For each exercise, a handout, diagrams, and instructor guide are provided.

**Exercise 1 (per group of two to four students):**

- A simulated "bag community" consisting of a lunch-size paper bag filled with different combinations of common objects such as beads, erasers, dry beans, etc.
- Graph paper
- Computers (allow for use of Excel spreadsheets) or calculators

**Exercise 2 (per group of two to four students):**

- Printout of sample community diagram A, B, C, or D (Word versions are included with the exercise)
- 1 Sharpie or other permanent marker
- 3 plastic transparencies, same size as the community diagram (8.5 x 11”)
- Data sheet (printed out, or electronically accessed on computer)

**Exercise 3:**

This is a thought activity. No special materials are required. A supplementary optional Powerpoint is included if the instructor wishes to project the diagrams during the activity.

**Student Version.**

[Exercise 1, Student Handout]
Exercise 2. Student Handout
Exercise 2. Student Data Sheet
Exercise 3. Student Handout

Instructor Version.
Exercise 1. Instructor Guide
Exercise 2. Instructor Guide
Exercise 2. Instructor PowerPoint Presentation
Exercise 3. Instructor Guide
Exercise 3. Instructor PowerPoint Presentation

Safety Issues.
None

Suggestions for Determining Student Learning.

I. Measurement of student learning in the classroom

These exercises were field tested in an undergraduate microbial ecology class. This was an upper-level biology course with 14 enrolled junior and senior biology and biochemistry majors. Prior to taking the course, all students had been exposed to a little ecology in general biology and some had more exposure in a previous ecology course. The exercises were done within the first month of the semester. Pre- and posttests were administered to students to measure what they had learned from the exercises.

At the end of the exercises an additional 20% of the students correctly identified the challenge of measuring and sampling for microbial presence and activity in the natural environment (65% of the total students). Identical results were obtained when students were asked to discriminate between measures of diversity (richness and evenness). Students were also 40% better at differentiating between species diversity and community structure. On the posttest all of the students reported strong agreement or agreement, that the activities helped them to understand difficulties in sampling microbial communities and differences between community structure and diversity.

When asked questions about experimental design 86 to 93% (it varied by question) of the students answered correctly on the posttest, compared with 28 to 50% on the pretest. Finally, before the exercises only one student was familiar with pseudoreplication and on the posttest 78% correctly defined this term. All but one of the students strongly agreed or agreed, that the activities helped them to understand how microbial diversity is measured and what it is that microbial ecologists do.

II. Assessment of usefulness of the activity (educators’ evaluation)

These exercises were also done at the American Society for Microbiology Conference for Undergraduate Educators (ASMCUE) in May 2008. The audience members were heterogeneous in background, training, and experience related to microbial ecology. After completing the three exercises, participants were given a survey to assess their opinions about the value of the activity for classroom use and how well it addressed the learning objectives.

The data clearly show that this activity is highly suitable for undergraduates in both microbiology and general biology courses and will also be helpful in teaching general ecology and statistics (Fig. 1). Respondents overwhelmingly agreed that this activity will help students appreciate considerations involved in the experimental design process and will reinforce important quantitative issues in biology. It is highly suitable for large lecture classrooms as well as the laboratory and can be combined with a wet lab or field experience (Fig. 2). Out of 48 sampled, 42 participants agreed that the use of primary literature in the activity was helpful; six said it was "somewhat helpful."

Overall, this activity was seen by microbiology educators as a very useful one for teaching the targeted principles.
FIG. 1. Suitability of activity for different student audiences, based on a survey of participants at ASMCUE 2008; $n = 47$. 
FIG. 2. Number of ASMCUE 2008 participants, \( n = 48 \), who agreed with the following statements:
(A) Will help students appreciate considerations of experimental design, (B) suitable for large lecture classroom, >40
students, (C) suitable for a laboratory exercise, (D) reinforces important quantitative issues in biology, (E) easily combined
with a wet lab or field activity.

References.


9. Ley, R. E., J. K. Harris, J. Wilcox, J. R. Spear, S. R. Miller, B. M. Bebout, J. A. Maresca, D. A. Bryant, M. L. Sogin, and


molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial


of Bland Findlay, Centre for Ecology and Hydrology, Dorset, UK)


Appendices.

Diagram Community A
Diagram Community B
Diagram Community C
Diagram Community D
Finding a pure culture of microorganisms in a natural environment is virtually impossible, as most microorganisms inhabit spaces also occupied by other species. The assemblage of species that occur together in an environment is called a community. Communities are important ecological units because they are different in organization and function than their individual species components. For example, food webs, nutrient cycling, and succession are all characteristics of communities. These characteristics will vary depending on the particular assemblage of species present. Therefore two important aspects of community ecology are the study of species interactions (predation, competition, parasitism, etc.) and species diversity.

To determine diversity of a community, an ecologist must decide upon the factors that will be used to distinguish members of the community. Most commonly we think of species diversity, the number of different species of organisms represented. Communities can also be divided up based upon other characteristics of their component organisms. Photosynthetic microorganisms in an aquatic community, for example, can be grouped according to the particular kinds of photosynthetic pigments they utilize. Each group would likely contain more than one different species of organism, but the group would share a common set of characteristics that set it apart from other groups. This is an example of dividing a community into operational taxonomic units (OTUs). OTUs are defined by the investigator of the community and depend on the question being asked. They are especially useful in communities where species identities are difficult to determine.

To acquire a good measure of diversity, communities must be sampled often enough that all of the different species or OTUs are detected, including those that are rare. So how many samples are enough? This will vary depending on how many species or OTUs are present in a community and in what numbers. Ecologists use a species sample curve to measure whether they have adequately sampled an environment. An example of such a curve is shown below (Fig. 1). The graph depicts the number of new species detected each time an additional sample is collected. When no more new species are encountered, the ecologist can be reasonably satisfied they have detected most of the species diversity in a sample.
FIG. 1. This graph shows that the more one samples, the higher the likelihood of “finding” all species present. At some point, in this case after about eight samples are collected, additional samples will no longer reveal additional species—and the line levels off.

Many times ecologists want to compare the diversity of multiple communities. To do this accurately, a quantitative measure of diversity is necessary. Diversity is described by two quantitative measures: species richness and species evenness. These concepts also apply to OTUs, but for simplicity we will refer to them in terms of species diversity. Species richness (Fig. 2) expresses the number of different species that occur in a community.

FIG. 2. The higher the species richness of a community, the greater its diversity. Community IV, for example, has the highest species richness, and by this measure alone would be considered most diverse of the eight communities represented.
Species evenness (Fig. 3) expresses the relative abundances of individual species within a community. For example, a community may contain 100 species (high species richness), but if one of these species comprises 90% of the individuals, that one species has a disproportionate influence on the functioning of the community.

![Graph showing species evenness of two sample communities](image)

**FIG. 3.** Of the two communities represented here, Community IV has higher species evenness and therefore higher species diversity.

Measures of species diversity that consider both species richness and species evenness are useful for comparisons of different communities. Such values provide a measure of the complexity of a community. Communities with higher values may have more complex and varied sets of interactions among member species.

One method for quantifying species diversity is the Simpson’s index. Simpson’s index (D) is represented by the following equation:

$$D = 1 - \left(\sum \frac{n_i}{N}\right)^2$$

where $n_i$ is the number of individuals of species $i$, and $N$ is the total number of individuals of all species. The calculation of $n_i/N$ is referred to as proportional abundance (p), because it is a measure of the proportional representation of each species in the community. These values are squared and then subtracted from 1, resulting in values that range from 0 to 1, with higher numbers representing higher diversity.

The following exercise demonstrates how this mathematical formula can provide us with a means for comparing the structure of different communities.
Sampling a community: how many samples are enough?

1. You have been given a “bag community.” Without opening the bag, record the letter identity of your community next to the heading for Table 1 on the Data Sheets.

2. Without looking into the bag, sample individuals, one at a time, from your bag community as many times as indicated by your instructor. As you draw out each individual from the community set it down beside you; do not return it to the bag.

3. Keep track of the number of times you sample your community and whether the type of individual (“species”) you sample each time is new using Table 1. Each time you collect a new species (one you have not yet encountered) from your community, indicate this by finding the row that corresponds to your sample number and enter the numeral 1 in the column labeled “Number of new species.” If you sample a species you have already drawn out of the bag previously, it is not a new species and you should write the numeral 0 in the “Number of new species” column. Keep track of the cumulative number of new species you sample from the community in the last column.

4. Once you have sampled your community the number of times instructed, construct a species sampling curve from the data you have collected using the sheet of graph paper provided to you. The x-axis represents the number of sampling events (e.g., 1 to 10), and the y-axis represents the cumulative number of species identified at each sampling event.

5. Look at your graph and determine whether you adequately sampled the diversity of your bag community. How can you tell if you did or did not?

Measuring diversity of a community: which one is more like the other?

1. Remove all members of the community from the bag and spread them out so you can see them. Group each “species” into its own separate pile. Determine the number of each species that you sampled and record these in the Simpson’s Index Data Table.

2. Determine the species richness of your community. Using the graph paper provided make a bar graph to demonstrate the species evenness of your community. Compare your measures of diversity to those of other groups in your class. Compared to others, did your bag community have high species richness? What about species evenness?

3. Now you can calculate a quantitative measure of diversity, which will allow you to compare the diversity in your community with those of other groups in the class. Remember that Simpson’s index (D) is represented by the following equation:

   \[ D = \Sigma \left( \frac{n_i}{N} \right)^2 \]

   where \( n_i \) is the number of individuals of species I, and N is the total number of individuals of all species. The calculation of \( n_i/N \) is referred to as proportional abundance (p) in the data table because it is a measure of the proportional representation of each species in the community. These values are squared and then subtracted from 1, resulting in values that range from 0 to 1, with higher numbers representing higher diversity. Determine proportional abundances and \( p^2 \) values for each of your species. Using the equation provided below the table, determine Simpson’s index of diversity (D) for your community. Compare this value to those your classmates have for their communities.
Data Sheet

TABLE 1. Species sample curve data for community

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Number of new species (0 or 1)</th>
<th>Cumulative number of species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td></td>
<td></td>
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<td>6</td>
<td></td>
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<td>7</td>
<td></td>
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<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2. Simpson’s index data table

<table>
<thead>
<tr>
<th>Species</th>
<th>Total number</th>
<th>Proportional abundance (p)</th>
<th>( p^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Proportional abundance is the number of individuals of a species/total number of individuals

Simpson’s index of diversity = 1 – (total of the \( p^2 \) values) = __________
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Exercise 2: Student Handout
Community Structure and Organization

Introduction

Characteristics of microbial communities that require special sampling consideration have already been mentioned in the general introduction for this activity. Exercise 1 introduces the concept of diversity and one of the ways to measure it after taking samples from a simulated community. The bag community is of course unrealistic in many ways. An important feature of the bag community is the way the items could be thoroughly mixed between each sample. Of course, in nature, microbes and other organisms are arranged, clumped, partitioned, etc., based upon many factors that affect them: availability of sunlight, nutrients, and water; interaction with other organisms; presence of antagonistic compounds or conditions; oxygen level and overall atmospheric pressure; and more. Organization and arrangement of cells will also vary temporally. Temporal changes may be rapid (exponential growth and competition in a rich broth, for example) or slow (seasonal or climate-based change).

In this exercise, you will consider how sampling plans are designed to help understand communities at the level of structure and organization, as well as species composition (diversity). The instructor will guide you through the activities.

Preexercise questions for discussion and review

1. What is a diversity index and what does it measure?

2. Write the formula for Simpson’s index of diversity, explain each of the variables, and also explain how the index is calculated.

3. List at least four characteristics of microbes that make the study of microbial diversity specially challenging.

4. When heading out to sample a microbial community, what factors will be important in the planning of your experimental design (brainstorming activity).

5. What is the size of the average prokaryotic cell? How do microbiologists detect and quantify prokaryotic cells? (List at least four general methods and briefly describe how they work.)
Step One
Your instructor will give you or your group a diagram of a sample microbial community. Before you see it, however (and remember that in the environment, microbes are typically invisible at the time of sampling), you will be asked to trace or draw three squares (representing sampling plots) on a transparency in a way that depicts your plan for sampling the community. With your partners, make a decision about how to lay out the squares, i.e., what sort of system or rules you will apply. Then, ask the instructor for your “community” diagram.

Step Two
Tape the transparency securely to your diagram and proceed to record data from each plot onto your data sheet. If you have a computer, data can be typed directly into the Excel spreadsheet, otherwise you will simply fill in the boxes on paper. Don’t forget to indicate the name of the community you have been assigned. Note that species are given letters as names and you will have to make decisions about separation of taxa. Your instructor will explain.

Step Three
Take a few minutes to look closely at your “community” and discuss the following questions within your group. Your instructor may choose to open the discussion to the class or ask you to turn in your responses as part of your assignment.

Take a look at the way the cells and/or organisms are arranged and answer the following questions:

a. How is this community different from one that might exist in the water column of a lake, for example?

b. If you were to imagine a habitat that this simulated diagram might represent, what would it be?

c. When sampling a community of this sort, does the scale of the area of investigation matter? In what ways will it affect your sampling approach?

d. If you would take data from nine small plots instead of the three relatively large plots you just did (adding up to the same comparable area), which sampling approach do you feel would give a more realistic or accurate picture of the actual community? What are the advantages and disadvantages of each approach? Do you think each method would give the same calculated Simpson’s index? Why or why not?

e. Finally, would a line transect approach be appropriate for sampling this community? Why or why not?

Step Four
Go ahead and prepare a new transparency, this time tracing or drawing nine small plots instead
of three large ones. Use the same system and rules that you used when laying out the larger plots. Proceed as before by recording plot data onto your data sheet.

**Step Five**
Draw three 6-inch transect lines on your third transparency. Before you do, establish rules for placement. Lay the transparency over the community and proceed to “walk” the transects, record the data for each one on your data sheet, as above.

**Step Six**
For each of your three sets of sampling data, calculate Simpson’s index of diversity. It will be helpful to use the Excel spreadsheet on your computer as a starting point. Do you obtain the same index using each of the sampling approaches? If there are differences, explain possible reasons why this occurred.

**Wrap-Up and Class Discussion**
Based on your findings, which sampling approach was more satisfactory in describing the actual community composition and structure? Note, Simpson’s index alone does not describe structure and organization.

**Sampling plots to trace**

For three large plots

![Diagram of three large plots]

For nine smaller plots

![Diagram of nine smaller plots]

For line transect:
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Exercise 2. Data sheet

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of individuals (count)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small plots</td>
</tr>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9</td>
</tr>
<tr>
<td>A</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>B</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>C</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>D</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>E</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>F</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>G</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>H</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>I</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>J</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>K</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>L</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
<tr>
<td>M</td>
<td>0.0  0.0  0.0  0.0  0.0</td>
</tr>
</tbody>
</table>

Total number of individuals counted: 0.0 0.0 0.0

Simpson's index for small plot: ____
Simpson's index for large plot: ____
Simpson's index for transect: ____
Exercise 3: Student Handout
Considering Replication

It is essential to consider the design of an experiment before setting out to collect data. Faulty experimental design can make it impossible to draw meaningful conclusions regarding the hypothesis being tested and can result in a waste of time and resources—valuable commodities in scientific research. Before an experiment is conducted the following should be determined: what, if any, factors (variables) will be manipulated, how will the factors be manipulated, and what are the appropriate methods for sampling and data collection.

In some cases a scientific investigation involves comparing responses to variable sets of conditions. For example, the effects of plant fertilizer on the abundance of nitrogen-fixing bacteria could be studied by applying different known concentrations of fertilizer to plots of agricultural land. Differences in the abundances of nitrogen-fixing bacteria before and after application of the fertilizer could be compared. The treatments in this experiment are the different concentrations of fertilizer. The control would be a plot of land that received no fertilizer. Good experimental design requires that the treatments and controls vary as little as possible in all factors except those that are manipulated, in this case fertilizer concentration. So for example, the experimental agricultural plots should vary little in the amount of water and sun they receive.

Where possible an experimental design should include replication. Replicates in an experiment should all be treated identically. They represent the natural background variation in a system. For example, a researcher interested in determining the number of viable bacteria in a body of water would not rely on counting bacteria once from a single sample of the water. Such an action could lead to faulty conclusions because that single sample of water might have fewer, or more, bacteria in it than another sample of the same volume. To account for such variance in the number of bacteria present it would be necessary to collect three, or preferably more, samples in identical fashion (replicate samples) and count the number of bacteria in each. These values would subsequently be used to calculate an average number of bacteria per unit volume of water for the habitat being sampled.

Statistics are used to evaluate the significance of differences detected between groups that receive variable treatment in an experiment. Most statistical calculations assume that replicate values are independent observations. This means that a single replicate value does not depend upon one other value more than it does on another. Each replicate should be treated the same as other replicates as much as possible. Using replicates that are not statistically independent is a form of pseudoreplication, a term coined by the ecologist Stuart Hurlbert in 1984 (1). Pseudoreplication also results from treating multiple measurements of the same sample as replicate measurements. An example is drawing multiple aliquots from a single flask of bacterial culture and referring to each
aliquot as a different replicate. An adequately replicated experiment would involve taking a single aliquot each from multiple flasks, all treated identically during the experiment. Pseudoreplication decreases our ability to draw accurate conclusions about what is happening in an experimental system. It does so by effectively lowering the sample size of an experiment and therefore decreasing chances that the natural background variation in a system will be adequately detected.

The series of word problems that follow is designed to help you think about the concept of replication in experimental design.

**Part I.**

A microbiologist is interested in testing how well extracts from three different plants (A, B, and C) can inhibit the growth of a single species of bacterium. The scientist plans to conduct her tests in shallow plastic wells. Into each well she will add equal volumes of plant extract and bacterial culture and incubate the cultures at the same temperature in a single incubator. She will compare growth after 24 hours by measuring turbidity in each of the wells using a spectrophotometer. She plans to replicate her experiment four times and sets it up as indicated in the diagram below. Each row of boxes indicates a single plastic dish, each of which contains four shallow wells.

PE refers to the plant extract solution taken from one of the three plants, A, B, or C. Water was used in place of a plant extract as a control.

What constitutes a treatment in this experiment?
Is there some aspect of the experimental design that could result in the experimenter finding differences and falsely attributing them to the treatment (plant types) when they are really caused by something else? What, other than treatment level, could result in differences in bacterial growth between treatments in this experiment? Explain your answer.

**Part II.**

The experiment testing the effects of plant extracts on bacterial growth was redesigned to remove the effect that a plastic dish might have on differences in bacterial growth between treatments. The new design is illustrated below.

Does the new design adequately address the problems posed by the previous experimental design? Explain your answer.

Are the replicates of the plant extract treatments independent of one another? Explain your answer.

Think of a better way to replicate this experiment. You are not constrained to the materials used above. You may suggest other ways of setting up the experiment; be creative. Draw your design and explain why this is a better method.
Part III.

Wastewater from urban centers is normally processed through a local wastewater treatment facility where solids are removed and steps are taken to reduce numbers of bacteria in the water. Treated water is often emptied into a local body of water; so it is not uncommon to find wastewater treatment facilities located beside rivers. An aquatic microbiologist investigating a river system decides to investigate whether the number of bacteria in the river is influenced significantly by the outflow from a wastewater treatment facility. In particular, the scientist is interested in bacteria commonly found in human intestines, many of which are gram-negative, lactose-fermenting rods collectively referred to as coliform bacteria. The microbiologist plans to collect water samples, return them to the laboratory, collect the bacteria on filters, and grow only the coliform bacteria using media selective for their growth. The number of coliform bacteria isolated from different locations in the river can be compared. Your job is to help the microbiologist design the experiment up through the stage where water samples are filtered and plated for isolation of bacteria. Describe how samples should be collected and used so that uniformity amongst replicates is ensured and pseudoreplication is avoided. Also describe an approach to the experiment that results in pseudoreplication and explain why.

Reference.
Using Microbial Ecology to Teach Experimental Design and Sampling Methods
Mary E. Allen and Ruth A. Gyure

Exercise 1: Instructor Guide
Measuring Diversity

Materials needed

- A simulated “bag community” for each group, consisting of a lunch-size paper bag filled with different combinations of common objects such as beads, erasers, dry beans, etc. Hints for filling bags: include about 20 to 40 objects in each bag, use different proportions and numbers of selected components, include at least one bag with a large number of types of objects evenly distributed and another bag with only one type of object.
- Graph paper
- Computers (to allow use of Excel spreadsheets) or calculators

Part 1. Sampling a community: how many samples are enough?
The bag communities can be assembled from a wide variety of materials, depending upon the goals of the instructor. If an instructor wants to discuss the concept of operational taxonomic units for example, they might define “beads” as an operational taxonomic unit and include in the bag communities a variety of bead shapes that would all be grouped together. Alternatively beads of different shapes could be counted as separate species. It is also useful to vary the size and texture of different items to represent phenotypic diversity in a community. For example, by using pieces of string, small balloons, small pencil erasers, etc.

Bag communities should vary in their species richness and species evenness so that groups can compare different measures of diversity with one another. The number of individuals per community can be determined by the instructor, but it is recommended that there be at least 20 items in each bag. It is sometimes useful to add a very rare or very small member to a community to demonstrate how difficult it is to detect every type of organism in a community.

Graph paper for making the species sampling curves is available online. Alternatively students can use computers to make their graphs. Student groups can be asked to share their curves with the rest of the class (e.g., drawing them on a blackboard) to demonstrate how differently communities may appear. This also provides an opportunity for discussion of the questions in Step 4. The class should be able to determine that the only communities that have been adequately sampled are those with curves that have leveled off. In some cases it may be desirable for the students to resume sampling their communities and discussing the way the species sampling curve changes as the number of sampling events increases.

This process takes about 10 minutes, depending on how many times a group samples a community. It is important to sample at least 3 to 4 times so that students begin to see the effect of sampling effort on the conclusions about diversity.
Part II. Measuring diversity of a community

Graph paper for making the species sampling curves is available online. Alternatively, students can use computers to make their graphs. Student groups should be given the opportunity to share their data with the rest of the class, for example making two big bar graphs on the blackboard that represent species richness and species evenness. Species richness, especially, is best understood when compared across different types of communities. This also provides an opportunity for discussion of the questions in Step 2.

A variety of diversity indexes can be calculated. Simpson’s index of diversity is used in this exercise because of its simplicity. Simpson’s index (D) is represented by the following equation:

\[ D = 1 - \left( \sum \left( \frac{n_i}{N} \right)^2 \right) \]

where \( n_i \) is the number of individuals of species \( i \), and \( N \) is the total number of individuals of all species. The calculation of \( \frac{n_i}{N} \) is referred to as proportional abundance (p) in the student version of the exercise, because it is a measure of the proportional representation of each species in the community. These values are squared and then subtracted from 1, resulting in values that range from 0 to 1, with higher numbers representing higher diversity.

See the example below. This part of the exercise takes approximately 20 minutes.
Sample calculations of Simpson’s index of species diversity

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Index: 0.83

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Index: 0.72
Exercise 2: Instructor Guide
Community Structure and Organization

Materials needed (per group of two to four students)
- Printout of sample community diagram A, B, C, or D (Word versions are included in exercise). These are necessary and must be printed in color.
- 1 Sharpie or other permanent marker
- 3 plastic transparencies, same size as community diagram (8.5 x 11”)
- Data sheet (printed out or electronically accessed on computer)

Introduction
Characteristics of microbial communities that require special sampling consideration have already been mentioned in the general introduction for this activity. Exercise 1 introduces the concept of diversity and one of the ways to measure it after taking samples from a simulated community. The bag community is of course unrealistic in many ways. An important feature of the bag community is the way the items could be thoroughly mixed between each sample. Of course in nature, microbes and other organisms are arranged, clumped, partitioned, etc., based upon many factors that affect them: availability of sunlight, nutrients, and water; interaction with other organisms; presence of antagonistic compounds or conditions; oxygen level and overall atmospheric pressure; and more. Organization and arrangement of cells will also vary temporally. Temporal changes may be rapid (exponential growth and competition in a rich broth, for example) or slow (seasonal or climate-based change).

In this exercise, students will consider how sampling plans are designed to help understand communities at the level of structure and organization, as well as species composition (diversity).

Time
Total time for this exercise is about 1.5 hours. The exercise can easily be done in less than an hour. Time will be longer or shorter depending upon how much time you choose for in-class discussion and data calculation. The questions and analysis may be assigned for students outside of class, some as preexercise questions for thought and others as postexercise follow-up questions. Another suggestion is to assign the questions as part of an online discussion group or blog (e.g., using Blackboard).

Instructor resources
- Instructor information (this sheet)
- Student handout with instructions for the exercise
- Student data sheet (Excel format)
- Four different community diagrams to print (use color)
- Optional: PowerPoint presentation
This can be used throughout the activity. Slides 1 to 10 can be used as an introduction to the exercise. Slides 11 to 15 can be used during the activity; there are projections of the diagrams that can be used when giving instructions. The final slides are helpful during follow-up discussion.

**Preexercise questions for discussion and review**

1. What is a diversity index and what does it measure?

   *This question helps reinforce what was learned in Exercise 1. Students should be asked to bring their notes with them from Exercise 1, or both exercises can be done during the same period in which case review is unnecessary.*

2. Write the formula for Simpson’s index of diversity and explain each of the variables and how the index is calculated.

   *It is always helpful to review exactly how the index is calculated. Doing this as a group in class assures that each student will be able to calculate the index properly when the exercise is completed, even if the calculation is part of the take-home assignment.*

3. List at least four characteristics of microbes that make the study of microbial diversity especially challenging.

   *These characteristics are given in the introduction to the overall activity. They include challenges of small size (direct visualization may not be possible), differentiation of prokaryotic taxa at species level, tight adherence to surfaces, interdependent relationships, problems of cultivation, and determination of ecological relevance. You and the students may be able to think of many more!*

4. When heading out to sample a microbial community, what factors will be important in the planning of your experimental design (brainstorming activity).

   *Here, help the students to understand that all researchers are limited by realistic constraints, such as how many samples one can afford. Trade-offs are made between the number of samples required in order to validly make comparisons (depends on standard deviation between replicates) and how much time and resource can be allocated. Other considerations include the size and scope of the project, the nature of the questions being asked, the scale of the study area, the nature and scale of interactions among organisms studied, the nature of the system (swirling water or static rock), the technological measuring capabilities available, the type of data that will be collected or measured in the lab if samples are being taken back, etc. This topic should stimulate rich discussion and the examples in the PowerPoint presentation will help to clarify and continue it at the end of class.*
5. What is the size of the average prokaryotic cell? How do microbiologists detect and quantify prokaryotic cells? (List at least four general methods and briefly describe how they are done.)

The average prokaryotic cell is between 0.1 and 10 microns, and unicellular eukaryotes are generally 10 to 100 microns—or about 10x larger. Microbiologists use microscopes to view cells directly, and even then, a variety of special techniques are required to sample, prepare, and label or stain the cells for meaningful observation and analysis. Cells can be counted in this manner, but often indirect methods of detection and quantification are preferred. DNA can be extracted from samples and several methods used to compare diversity by PCR amplification and/or enzymatic digestion (DNA “fingerprints”). For a more detailed description of current techniques for measuring prokaryotic diversity, I recommend the article by Vigdis Torsvik (3). Torsvik was one of the first microbial ecologists to apply molecular genetic techniques to the comparative study of microbial communities.

Guiding Students through the Steps of the Exercise

Step One
I recommend that you have students work in groups. Pass out transparencies, markers, and handouts first and do not pass out community diagrams. At this stage you may use the PowerPoint to project an image of one community so that students understand what to expect. In order to establish sampling “rules,” students will need your help in deciding how to establish plots.

A simple approach for this activity will be to randomly place squares or frames onto the sampling area. In our case, students will overlay their black clear transparencies on the diagram of a sampling area. In the field, ecologists carry flexible wire, plastic, or wooden frames and toss them out into the sampling area in a randomized way.

It is a very good idea to discuss other more rigorous methods with students. For example, a very common and valid approach is to measure out the entire sampling area into blocks of equal size, number them as in a grid, then use a random number generator to select a subset.
that can be reasonably sampled given time and resource availability. Note that plots of equal size like this are also referred to as quadrats, see definitions below.

In ecology a plot is a term used to describe a geographic space from which data are collected. Plots can be of varying size and shape, depending on the goals of the study. Ecologists typically collect data from plots of known sizes, referred to as quadrats—which can be any size or shape. If an area is too large for data to be collected throughout, a grid may be overlaid on the plot and data collected within randomly selected sections of the grid. Below is an example of a grid that may be overlaid on a sampling area. Each square can be numbered, then some are randomly selected as plots, in this case also quadrats, to be sampled. In the field, ecologists often walk out plot lines with a tape measure if this approach is utilized. Plots can also be defined by GPS coordinates. Imagine the following grid labeled with numbers from 1 to 25; five plots are randomly picked to be sampled. Random numbers can be drawn from an envelope of 25 pieces of paper or selected using a random number generator such as those found at http://stattrek.com/Tables/Random.aspx or other publically available Internet sites.

Step Two
The distinction of “taxa” will involve student judgments about color, shape, and size. It is worth bringing up the point that this may work for a simulated community on paper but is quite inadequate in defining prokaryotic taxa in nature. This is a good time to review how prokaryotic taxa (at the species level) are distinguished! There is a PowerPoint slide that addresses this, and I also call your attention to the article by Cohan (1).

Steps Three and Four
a. How is this community different from one that might exist in the water column of a lake, for example?

In the water column of a lake, organisms are not stably arranged. Water currents and transient temporal changes (e.g., diurnal sunlight) will change the location of individuals and clusters, sometimes randomly. Attached organisms are more permanently organized spatially and temporally. Changes will be reflected metabolically but not in community composition or structure.
b. If you were to imagine a habitat that this simulated diagram might represent, what would it be?

_Some of the simulated communities are complex and dense; others are simpler and less dense. Soil communities would have a high degree of complexity (diversity) with perhaps clumped patterns in arrangement of cells. Bacteria on the surface of a catheter will be less diverse and perhaps more predictably arranged, maybe along a gradient of nutrient availability or temperature. By giving examples of this sort, you will help students brainstorm about communities they can imagine or may encounter and the diversity and structure they expect to be associated with such communities._

c. When sampling a community of this sort, does the scale of the area of investigation matter? In what ways will it affect your sampling approach?

_Scale most certainly matters. Even though many microbes are particle attached and arrange themselves in predictable ways on the surface of the particles, this scale of organization is almost never of interest when studying larger-scale issues of ocean, lake, river, or soil diversity. For example, soil moisture, temperature, and organic content will drive large differences in microbial community composition and structure when different agricultural areas are compared. Small scale heterogeneity within each of these samples is irrelevant in that it does not contribute to the variation of interest._

d. If you would take data from nine small plots instead of the three relatively large plots you just did (adding up to the same comparable area), which sampling approach do you feel would give a more realistic or accurate picture of the actual community? What are the advantages and disadvantages of each approach? Do you think each method would give the same calculated Simpson’s index? Why or why not?

_Students will hopefully observe that by using several smaller plots, they are more likely to realistically assess community structure, especially in a heterogeneous environment. However, the diversity index may not differ significantly with plot size. If student groups do not all observe and draw these conclusions, you can use the PowerPoint presentation Part I to show the Sample Data set which does illustrate these points. In the final wrap-up with PowerPoint presentation Part II, you will see there is a very clear example from the literature (2) in which this sampling hypothesis is tested._

e. Finally, would a line transect approach be appropriate for sampling this community? Why or why not?

__See the transect discussion below before initiating this topic with students. They will have some ideas at this point and will be in a better position to discuss transect sampling as compared to quadrats after they have actually done it.__
Step Five

A standard method for transect sampling is called the “point method.” One determines a number of points, distributed randomly or regularly in the survey area. For example, these may be the intersecting points in the grid students established earlier. Randomly chosen coordinate pairs can be used to define transect lines. In discussion of transects, it should be pointed out that for microbes, samples are taken along the transect, but identification is not done in the field as would be done with plants. The same considerations that were made in plot-quadrat sampling should also be taken into account when determining line transect length, placement, and sample frequency along the transect.

Line transects are perhaps most useful when aligned along a known environmental gradient. In lakes, depth sampling is basically vertical transect sampling and is highly appropriate based upon known layering of communities vertically in the water column. Other common transect scenarios include sampling along a gradient of altitude or with distance from a point source of contamination. In these cases, placement of the transect addresses the research question or hypothesis and is not random.

Step Six

For each of your three sets of sampling data, calculate Simpson’s index of diversity. It will be helpful to use the Excel spreadsheet on your computer as a starting point. Do you obtain the same index using each of the sampling approaches? If there are differences, explain possible reasons why this occurred.

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Wrap-Up and Class Discussion

Based on your findings, which sampling approach was more satisfactory in describing the actual community composition and structure? Note, Simpson’s index alone does not describe structure and organization.

At the end of this exercise, it may be useful to use the PowerPoint presentation Part II to help students review the concepts learned and point out some actual studies in the literature.

The PowerPoint slide illustrating the Fenchel and Findlay sampling experiment is informative when students are comparing all of the data sets. They tested different sampling approaches based on quadrat size and number (in this case cylindrical). It will be interesting to note whether or not students’ data aligns with their findings.

References.


REVIEW
Considerations When Sampling Microbes in the Natural Environment:
1. Detection and visualization
2. Definition and differentiation of taxa
3. Cultivation issues and ecological relevance
4. Interdependence (consortia)
5. Adherence (biofilms)

WHAT IS A PROKARYOTIC SPECIES???
Current, genetic view of species-level difference:
DNA-DNA hybridization rate of about 70%
16s rDNA similarity of >98%
CAN I ISOLATE?
IS THE ISOLATE ECOLOGICALY RELEVANT?

There is a wide diversity of organisms in most environments! However, when plated on standard growth media in the lab, very few of these species will grow. Even if an isolate is obtained, how do we know it is one that plays an important role in the system?

BIOFILMS

• Elude traditional sampling and visualization methods
• Cells tightly clumped and difficult to separate, isolate, identify
• Phenotypic variation difficult to assess after sampling

What is a biofilm? Biofilms are microbial communities that are attached to an environmental surface. They usually encase themselves in an extracellular polysaccharide or slime matrix. Biofilms may be found on any environmental surface where sufficient moisture and nutrients are present. Their development is most rapid in flowing systems where adequate nutrients are available.

An excellent educational guide to biofilms may be found at:
http://www.biofilmsonline.com/cgi-bin/biofilmsonline/ed_intro_primer.html

S. Lowry—University of Ulster—Stone/Getty Images

Diversity: species composition, i.e., how many different species are there, and how are the numbers distributed among them.

Structure: how are these individuals distributed and organized in the environment?

These simplistic diagrams illustrate some possible ways in which organisms may be distributed in the environment. Consider these groupings at smaller and smaller scale!

Actual distribution will show combinations of these patterns, and will also differ depending upon the scale at which you observe, sample, and measure it.
BRAINSTORM: HOW DOES ONE MAKE DECISIONS ABOUT SAMPLING?

1. Size of population, community, or system area of interest?
2. Scope of study?
3. Budget?
4. Variability (standard deviation, error)? Depends on heterogeneity; abundance; distribution, both spatial and temporal; method, etc. (see link below for a great discussion about statistical considerations).
5. Technological ability (Can one directly observe organisms or cells? Can one target individuals or groups with specificity? How precise are the units of measurement?)
6. Experimental approach
7. Scale! (many issues)

STATISTICAL METHODS AND PRIMER, ENVIRONMENTAL APPLICATIONS:
http://epa.gov/bioindicators/statprimer/

Sampling soil microbes in a relatively static soil community
Even here, there are challenges of scale and dealing with localized heterogeneity that could mask larger scale changes of interest.

EXERCISE 2:

Let's “sample” some diagrammatic representations of microbial communities!

In this exercise, sampling and measuring diversity alone is not the goal. We would also like to sample in a way that informs us about the distribution of organisms present.

Here is an example of one community that may be assigned.
BEFORE YOU RECEIVE A COMMUNITY, YOU MUST DECIDE ON A PLOT SAMPLING SCHEME.

STEP 1. Trace your three large plots onto the transparency after deciding on a placement plan. When finished, ask the instructor for your sample community.

STEP 2. Record the number of individuals for each species observed in each plot on your data sheet.

STEP 3. Discuss the following questions as a group, as a class, or later as a homework assignment.

a. How is this community different from one that might exist in the water column of a lake, for example?

b. If you were to imagine a habitat that this simulated diagram might represent, what would it be?

c. When sampling a community of this sort, does the scale of the area of investigation matter? In what ways will it affect your sampling approach?

d. If you would take data from nine small plots instead of the three relatively large plots you just did (adding up to the same comparable area), which sampling approach do you feel would give a more realistic or accurate picture of the actual community? What are the advantages and disadvantages of each approach? Do you think each method would give the same calculated Simpson’s index? Why or why not?

e. Finally, would a line transect approach be appropriate for sampling this community? Why or why not?

STEP 4. Repeat the sampling this time tracing nine small plots rather than three larger ones. You must use same rules for the placement scheme! Record data as before.

STEP 5. Draw three transect lines and determine a placement scheme. Record data.

STEP 6. Calculate Simpson’s index of diversity for each of the three sampling approaches.

PART II. Postexercise discussion and review

In the next three slides, you will see what the four communities actually looked like and an example of how the three sampling schemes might be applied to Communities C and D, with a sample data set for class discussion.
### Sample Data Set

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**FINAL THOUGHTS**
TRANSECTS

Our exercise here focused mainly on plot sampling.

When is use of transects appropriate?

• For example: when one hypothesizes a gradient relationship and wishes to test it using regression analysis, see below!

• In this study, researchers looked for change in numbers of fecal indicator organisms across various transects in relation to the water’s edge.

• Keep in mind, most aquatic depth sampling is, in effect, transect sampling.

Concluding Thoughts

• The question being asked drives the experimental design.

• The practical limitations of sampling limit the type of question that can be asked—and answered!

• Pilot sampling is always essential to assess the nature of the system!!

We will better understand the scientific process when we appreciate the considerations that go into every scientist’s sampling and measurement plan!
EXERCISE 3: INSTRUCTOR GUIDE
CONSIDERING REPLICATION

Part I.
This section should take approximately 10 minutes.

What constitutes a treatment in this experiment and how many different treatments are there?  The treatments in this experiment are the different plant extracts (PE), so there are three treatments: PE A, PE B, and PE C.

Is there some aspect of the experimental design that could result in the experimenter finding differences and falsely attributing them to the treatment (plant types) when they are really caused by something else?  What, other than treatment level, could result in differences in bacterial growth between treatments in this experiment?  Explain your answer. Yes, if there are differences between individual plastic dishes that affect bacterial growth, this could result in measurable differences between groups of treatments. The experimenter could attribute the variability in results to effectiveness of plant extracts, when the variability could instead be due entirely, or in part, to differences in the individual plastic dishes. In other words the conclusions of the experiment would be false.

Part II.
This section should take approximately 15 minutes.

What constitutes a replicate in this experiment?  The replicates are the four wells in a single plate. Each well in a plate contains exactly the same materials—extract from the same plant (A, B, or C) and bacteria—so they are each replicates of an experimental treatment.

Are the replicates of the plant extract treatments independent of one another?  Explain your answer. No, the replicates are not independent of one another because they are not all treated the same. For example, the association of PE A with PE B in the same dish might influence the outcome of the experiment more than the association of PE A with PE C (together in a different dish). Therefore the replicates of PE A are not independent because one may depend more on its association with PE B than one not as closely associated with PE B.

Think of a better way to replicate this experiment. You are not constrained to the materials used above, you may suggest other ways of setting up the experiment; be creative. Draw your design and explain why this is a better method.  Answers to this question will be variable. Students should take into consideration that all treatments and the control should experience the same set of conditions and that replicates should be independent. Below is an example of an experimental design that uses plastic dishes with
five wells. Each plastic dish contains all four treatments and a control, so the dishes themselves will not impact the results of one treatment more than another. The replicates are independent of one another because they are each randomly assigned to a spot in each dish. Students may suggest setting the experiment up in test tubes or flasks, in which case they should consider placement of these in a test tube rack or an incubator. The placement of different treatments and replicates should follow similar guidelines as those used to assign them to wells in the dishes below.

Additional notes.

As part of the experimental design, students might also consider the source of the bacteria for the experiment. If all of the bacteria are taken from a single flask, this would constitute a form of pseudoreplication, where multiple measurements of the same sample are treated as replicate measurements. To adequately replicate the experiment, treatment wells should be inoculated from four different flasks of bacteria. Culture from one flask would be used to inoculate one set of wells of each treatment plus one control (one PE A well, one PE B well, etc.), while culture from a separate flask would be used to inoculate the second set of treatment and control wells, etc. This ensures that the results of the experiment are applicable to more than a single flask of bacteria. Especially motivated students might ask how many generations each flask of bacteria should be separated by to ensure they represent the true genetic variation of the bacterial population being investigated.
Part III.

This section should take approximately 20 minutes.

The task assigned to the students at the end of the experimental scenario is purposefully not specific. It is meant to encourage students to integrate what they have learned from Parts I and II with a more complete consideration of pseudoreplication. Students should be encouraged not to focus too much on the smallest details of the sampling and methods for isolating bacteria. Rather, they should focus their attention on a good experimental design. For students less familiar with experimental design and microbiology, it might be helpful to provide some leading questions. Examples include: From where should water samples be collected? How many samples should be collected from each site? What will constitute a replicate sample? How can statistical independence of these replicates be ensured?

The experiment can be designed in a variety of ways. Listed below are some considerations to ensure the question is addressed and replicates are uniform and independent.

• Water samples should be taken from a site at the wastewater treatment outflow and upstream of this site so that the influence of the outflow on numbers of antibiotic-resistant coliform bacteria can be compared to normal background levels. Students may also propose sampling downstream of the outflow, which is a good idea as it indicates how localized the effect of the outflow might be. The number of upstream and downstream sampling sites may vary; the final decision will depend on resources (time and supplies), but obviously more sampling sites allow the experimenter to draw broader conclusions.

• Once the location of sampling sites is determined, the number of samples collected from each site should be determined. These will ultimately provide the replicates of the experiment, so at least three should be collected, as this is the minimum number necessary to compute an average. Obviously, having more replicates is generally better.

• A single aliquot from each water sample should be filtered for collection and isolation of bacteria. The number of coliform bacteria isolated from this aliquot of water constitutes one replicate value. The average number of bacteria isolated from all of the replicates taken from a single site can be compared to an average value for a different site.

• A common pitfall is to suggest taking a single water sample from each site on the river, returning this sample to the laboratory and withdrawing multiple aliquots from it for filtration and isolation of bacteria. This is pseudoreplication because multiple measurements of the same sample are treated as replicate measurements, which they are not.
EXERCISE 3

These slides can be projected to aid in discussion with students. The diagrams are duplicated in the student activity sheet.

EXERCISE 3
PART 1.
EXERCISE 3
Part 2.