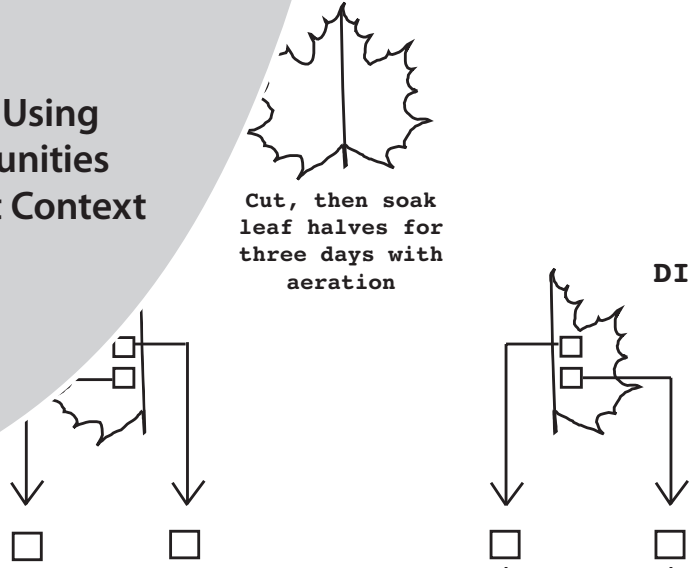
INQUIRY & INVESTIGATION

Teaching Basic Lab Skills Using Diverse Microbial Communities in a Biologically Relevant Context



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Abstract

We present a laboratory-based exercise that is used to teach basic lab skills (e.g., aseptic technique and enumeration) using naturally occurring microbial communities in a real biological context. Students examine the colonization by microbial communities of leaves that fall into streams. Leaf decomposition reflects enzymatic activity by microorganisms such as aquatic fungi and bacteria and maceration by invertebrate shredders. The microorganisms help facilitate the cycling of nutrients and energy in the stream's ecosystem. This exercise effectively teaches students to use lab skills to quantify microorganisms found in nature, investigates groups of microorganisms involved in leaf degradation in streams, and stimulates interest in both microbiology and ecology.

Key Words: *Microbial communities; microbiology lab skills; fungi; cellulose-degrading microbes.*

Traditionally, introductory laboratory courses that use microbiological techniques have a medical focus and tend to use pure cultures to teach basic lab skills (Leboffe & Pierce, 2006). Consequently, students get comparatively little exposure to the dynamics of microbial communities, especially in natural biological contexts. However, microbial species often live in complex multispecies communities in both medical and environmental contexts. This laboratory exercise provides students the opportunity to learn basic lab skills while working with diverse microbial communities. Specifically, students examine the colonization of leaf material in streams by naturally occurring communities of microorganisms. This colonization plays a significant role in ecosystem dynamics because it facilitates energy flow and nutrient cycling in streams (Graça, 1993).

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When leaves first enter streams they undergo leaching that releases dissolved organic and soluble inorganic materials from them (Webster & Benfield, 1986). The leaves are then colonized by multiple species of bacteria and fungi, often forming microbial biofilms (Bärlocher & Kendrick, 1975; Suberkropp & Klug, 1976; Graça, 1993). Biofilms, which are common in nature, are microbial communities covered in exopolysaccharide material; they are often visible as slimy films on wet surfaces (e.g., on wet rock surfaces near the stream's edge; Atlas & Bartha, 1998). Biofilms form as a sucession of different organisms colonize the leaf-litter surface. Within hours of falling into the stream, leaf-litter particles are colonized by bacteria, and other groups of microorganisms appear later. Temperature and other environmental factors influence the rate at which the leaf litter is colonized (Atlas & Bartha, 1998).

Microorganisms colonizing leaf litter use enzymes to break down complex molecules in the leaf, such as cellulose, chitin, and lignins (Webster & Benfield, 1986). Bacteria involved in leaf decomposition include the Actinomycetes (e.g., *Streptomyces*, *Nocardioides*, *Pseudonocardia*, *Nocardia*, and *Micromonospora*) and bacteria from the *Cytophaga-Flavobacterium-Bacteroidetes* group (Wohl & McArthur, 1998; Lydell et al., 2004). Fungi involved in leaf decomposition include aquatic hyphomycetes such as *Clavariopsis aquatica*, *Tricellula aquatica*, *Tripospermum camelopardus*, and *Gyoerffyella rotula* (Descals, 2005; Gulis et al., 2005). These microorganisms play a critical role in the degradation of leaf material by both digesting the leaf matter and increasing the palatability of the leaf material to macroinvertebrates (e.g., isopods and amphipods; Webster & Benfield, 1986). In addition, these microorganisms produce biomass that is consumed by other organisms in the community. Thus, microbial colonization facilitates the decomposition of leaf material and provides food for other organisms within the ecosystem.

In the exercise presented here, students measure colonization of leaf material by both bacteria and fungi during the first 3 days of leaf submersion using serial dilution, aseptic technique, bacterial plate spreading, and both fungal and bacterial staining to quantify microbial colonization of leaf material. This exercise can be completed in two lab sessions, is relatively inexpensive, and does not require any prior experience with microbiological techniques. In addition, both qualitative and quantitative approaches can be used to describe the data, which gives instructors the opportunity to modify the exercise according to the statistical expertise of the students.

To date, we have used a somewhat recipe-based approach to run this exercise. Here, we introduce a more inquiry-based approach, in which students are given the opportunity to generate new hypotheses and design experiments after completing the initial experiment (see Discussion). It may also be possible to modify the experiment so that a more inquiry-based approach is used for the entire exercise. For example,

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the instructor could provide the students with the relevant background information on the biology of the system, describe the materials that will be available (i.e., presoaked leaves, stream water, etc.), then ask the students to generate testable hypotheses and workable experimental designs. Alternatively, the students could be provided with the experimental design and asked to generate their own flow chart that summarizes the design (see Figure 1 for an example).

○ Experimental Procedure

The students are placed into groups of two to four and asked to collaborate on the assignment. The procedure is summarized in Figure 1. Unless otherwise stated, all chemicals can be obtained from Fisher Scientific. The following is a detailed description of the exercise.

Leaves should be obtained during the fall as soon after abscission as possible. In our experiments we have used maple leaves (*Acer* spp.) collected within a couple of days of falling. The leaves can be autoclaved (121°C, 25 minutes, dry cycle) to kill any microorganisms that may be present. The autoclaving process uses steam at high temperature and pressure, and as a result, the leaves will be fairly brittle but will remain intact when handled with care. Several layers of leaves separated by parchment paper can be autoclaved at once, then allowed to air dry overnight before being frozen at −20°C. Stacks of individual leaves, separated by parchment paper, are placed in clean brown paper bags and frozen until use.

Three days before the first lab session, complete leaves should be cut into two halves. Leaves are cut along the midvein, rather than using separate leaves, to avoid potentially confounding effects associated with preexisting variation in individual leaves. Each leaf half is assigned to one of two containers (e.g., round plastic containers, 18 cm diameter, 8 cm height). These containers are partially filled (~400 mL) with distilled water or stream water and the leaf halves are completely submerged in the water. Once the leaf halves are in the containers, a piece of 1-mm mesh (30 × 30 cm section of screen mesh) is placed over each container so that the middle region of the mesh is submerged in the water. For the stream-water treatment, detritus (leaf material and twigs) and sediment collected from the stream are laid on the mesh so that the microorganisms present in the sample are in proximity to the experimental leaf. Microorganisms (bacteria and fungal spores) from the stream water and detritus will then colonize the leaf material. For the distilled-water treatment, no additional material is placed on the mesh. The leaf halves are held in these containers for 3 days at room temperature and the water is aerated using an air pump (available from a pet supplier).

During the first lab session, the students use sterile forceps, gloves, and scissors to cut two squares (1 × 1 cm) from each leaf half (see Figure 1). To minimize error associated with cutting the squares, we provide the students sterile mesh squares (1 × 1 cm of 1-mm mesh) that can be placed on the leaf to guide the cutting. Two leaf squares are needed for each treatment (distilled vs. stream water). One square will be used to measure fungal colonization and the other will be used to measure bacterial colonization.

Fungal Colonization

During the first lab session, the students mount leaf squares in a few drops of lactophenol cotton blue stain (20 mL phenol [Sigma-Aldrich, no. P9346], 20 mL lactic acid [Sigma-Aldrich, no. W261106], 40 mL glycerine [Sigma-Aldrich, no. S362158], 0.05 g aniline blue [Sigma-Aldrich, no. 415049], and 20 mL distilled water, following Hanlin & Ulloa, 1988) and quickly pass the slide through the flame of an alcohol lamp twice to set the stain. After cooling, a cover-slip is placed over the stained leaf. The students examine the squares using standard compound light microscopes at 40× magnification. The students observe 10 fields of view and record the presence (+) or absence (−) of fungi in a data sheet (Figure 2A). The fields of view are observed systematically in three rows of three or four fields, moving in a left to right direction, to avoid recounting any leaf regions. Fungal colonization can be identified because fungal cells (hyphae) stain a deep blue. We have found that it is helpful to set up demonstration microscopes of stained leaves inoculated with fungi, to help the students distinguish between the stain and stained fungal tissue. Commercial preparations of lactophenol cotton blue are also available (Fisher Scientific, no. R40028).

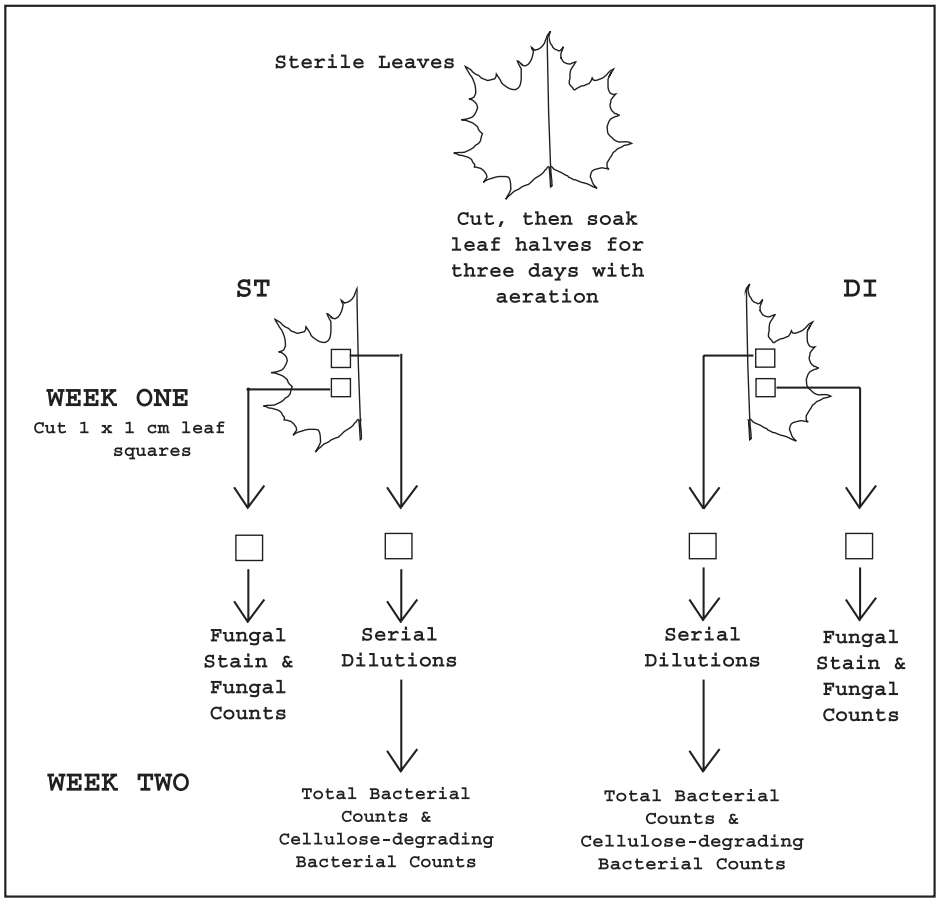

Figure 1. A flow chart of the laboratory exercise (DI = distilled water, ST = stream water).

Bacterial Colonization (Total Bacteria & Cellulose-Degrading Bacteria)

Prior to the first lab session, carboxymethylcellulose agar plates should be prepared (12 per student group) according to the following recipe to make 3 L of agar (120 plates, for a class of 30 students): 15.0 g carboxymethylcellulose, 3.0 g $NaNO_3$, 3.0 g K_2HPO_4, 3.0 g KCl, 1.5 g $MgSO_4$, 1.5 g yeast extract, 3.0 g glucose, 51.0 g agar, and 3 L water (Apun, 1995). This medium is used to detect cellulose-degrading bacteria. The carboxymethylcellulose can be obtained from ICN Biomedicals (no. 101278).

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A Fungal Counts (Fields of View)

(Scored as + or – for presence of fungi)

	1	2	3	4	5	6	7	8	9	10	Total
ST											
DI											

B Bacterial (Colony) Counts

	Total Bacteria		Cellulose-Degrading Bacteria	
Dilutions	ST	DI	ST	DI
10^{-2}				
Mean				
10^{-3}				
Mean				
10^{-4}				
Mean				
10^{-5}				
Mean				

Figure 2. Sample data sheet for fungal and bacterial (colony) counts. **(A)** Students observe 10 systematically chosen fields of view using a light microscope (40×) and record the presence (+) or absence (−) of fungi for each observation. **(B)** Students count the total number of bacterial colonies and the number of cellulose-degrading colonies on each agar plate at each dilution of sample (DI = distilled water, ST = stream water).

Students typically work in groups of three for this exercise. They must be careful to practice sterile technique when handling the leaf squares and preparing the serial dilutions. During the first lab session, the students use a sterile pestle to grind the leaf square in 1 mL sterile distilled water for 2 minutes at room temperature. Volumes of the leaf extract are then used to prepare serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of the extract. Using sterile pipette tips, the entire ground leaf suspension is then transferred to a bottle containing 99 mL sterile water and thoroughly mixed, forming a 10^{-2} dilution. One milliliter of the 10^{-2} dilution is transferred to a fresh tube with 9 mL of sterile water and mixed, forming a 10^{-3} dilution. Further serial dilutions of 10^{-4} and 10^{-5} are prepared by transferring 1 mL of the 10^{-3} and 10^{-4} dilutions, respectively, into fresh tubes with 9 mL of sterile water. Throughout the preparation of the dilutions, the students should flame the mouth of the tube before and after adding the leaf suspension (unless using plasticware).

Carboxymethylcellulose agar plates are inoculated with 100 µL of one of the four suspensions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}). The suspension should be carefully spread over the entire surface of the agar plate with a "hockey stick" spreader that has been sterilized by dipping in ethanol and flaming. A diagram of this spread-plate setup can be found in a standard microbiology laboratory manual (e.g., Cappuccino & Sherman, 2002). If one wishes to avoid the use of ethanol and flame, disposable sterile spreaders can also be used (Remel, no. R4853000). Three plates are used for each dilution to provide replicate measures. The plates are then incubated in an inverted position (to avoid condensation effects) for 1 week at room temperature.

During the second lab session, the students record the number of bacterial colonies (using the data sheet provided in Figure 2B) then calculate the average colony number per sample for each dilution (using counts obtained from the three replicate plates). If the number of colonies present on a plate is sufficiently high that enumeration is not possible, they should be scored as TNTC (too numerous to count).

Once the colony counts have been completed, the students quantify the number of cellulose-degrading bacteria present by flooding the surface of the agar plates with 10 mL of 1 mg/mL Congo red stain (ICN Biomedicals, no. 15071125) for 15 minutes and then flooding them with 10 mL of 1 M NaCl for 10–15 minutes. Colonies of cellulose-degrading bacteria are identified by the appearance of a clear zone around the colony that results from digestion of the carboxymethylcellulose by the bacteria. The students record the number of cellulose-degrading colonies per plate and calculate the average colony number per sample for each dilution (using the three replicate samples).

○ Sample Data & Analysis

A sample data set for the exercise is available on request. Using both qualitative and quantitative measures, it is apparent that leaves exposed to stream water were colonized rapidly by fungi and bacteria whereas leaves exposed to distilled water were not. In addition, cellulose-degrading bacteria were common on the stream-water leaves, accounting for approximately 17–20% of the bacterial community present, but were rarely present on the distilled-water leaves. The results demonstrate clearly that leaf material was colonized by a diverse community of microorganisms (fungi, cellulose-degrading bacteria, and non-cellulose-degrading bacteria) and that organisms were abundant in the stream water (and detritus) obtained from the local stream.

In addition to demonstrating microbial colonization and community diversity, the students can also gain valuable experience in both data analysis and interpretation. For example, the students can be encouraged to run their own statistical analysis. For the quantitative data, a paired t-test could be used on both the fungal and bacterial counts (using Microsoft Excel). These analyses would be paired because each student group worked with two halves of the same leaf, which controls for potential effects of preexisting variation in leaf quality.

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The students can also be asked to discuss how using different leaves (rather than leaf halves) could have influenced the results. Finally, the students can be asked to determine which dilution provided the best estimate of bacterial colonization. In our case, the 10^{-2} dilution was excluded because most of the plates were scored as TNTC. We would also recommend excluding the 10^{-5} dilution because colony counts were low and, hence, more prone to sampling error than dilutions with large colony counts. In our example, the remaining dilutions (10^{-3} and 10^{-4}) could be used to estimate population size. These values yielded estimates of 7.2×10^6 (obtained from the 10^{-3} leaf dilution) and 21×10^6 (obtained from the 10^{-4} leaf dilution) bacterial colony-forming units per milliliter (average $\approx 14 \times 10^6$). Consistent with this interpretation, microbiologists often adopt a "30−300" rule, whereby counts are made only on plates with more than 30 but less than 300 colonies. By plating and counting the four dilutions presented here, students will obtain a conceptual understanding of a rule that may at first seem somewhat arbitrary.

○ Discussion & Conclusions

In performing this laboratory exercise, students should acquire basic lab skills while simultaneously gaining a greater appreciation for the critical role that microorganisms play in both energy flow and nutrient cycling in nature. Using this starting point, students can be encouraged to think about other ways that microbial communities influence relationships in nature. For example, the gastrointestinal tract of humans is home to a vast community of microorganisms (Hooper & Gordon, 2001), and diverse communities of bacteria and fungi are used in the management of wastewater to break down the suspended organic matter, dissolved organic material, and inorganic compounds in sewage (Atlas & Bartha, 1998). These microbial activities contribute to the release of mineral nutrients and organic humus into the water supply. Students could also be asked to consider the impact of microbial communities on health and disease in humans. For example, diverse communities of bacteria contribute to plaque and tooth decay, and bacteria that naturally occur on the body (normal flora) help prevent infection by pathogenic bacteria (Madigan et al., 2000).

This laboratory exercise gives students a relatively simple glimpse into a complex world of microorganisms that may at first seem somewhat intimidating. Ideally, this type of exposure will spark sufficient interest in the students that they will be motivated to learn the more technical skills required to study microbial community dynamics at an advanced level. This exercise is also beneficial because it can be used to emphasize the integrative nature of modern science. For example, leaf degradation in streams is dependent on both the colonization of leaf material by microorganisms (microbiology and microbial ecology) and the feeding behavior of macroinvertebrates (animal behavior and behavioral ecology). The combined actions of these organisms result in the release of energy and nutrients (aquatic biology and ecosystem ecology), which helps to sustain a diverse stream community (population ecology and community ecology). Students could be encouraged to explore these areas as well as the connections between areas by posing questions and designing experiments that follow from the exercise presented here.

For example, students might use the basic skills they have acquired in the experiment to identify the relative contributions of the different sources of microorganisms to the microbial community that colonized the leaves. In the exercise presented here, stream water, detritus, and sediment were included as potential sources of microorganisms. Students could design a relatively simple experiment in which they expose fresh leaves to various combinations of these sources and then quantify the microbial colonization associated with each source.

Students could also take a more behavioral approach by examining the effects of microbial colonization on the feeding behavior of macroinvertebrates. Previous studies have shown that microbial colonization of leaf material influences the feeding behavior of macroinvertebrates (Graça, 1993). Specifically, colonized leaves are more palatable than uncolonized leaves and are preferred over uncolonized leaves in diet preference trials (Graça, 1993). Students could be encouraged to run an experiment in which they expose macroinvertebrates (e.g., aquatic isopods or amphipods) to colonized and uncolonized leaves and then record feeding activity on the different leaf types (for examples of this type of experiment, see Canhoto et al., 2005; Sparkes et al., 2008).

○ Acknowledgments

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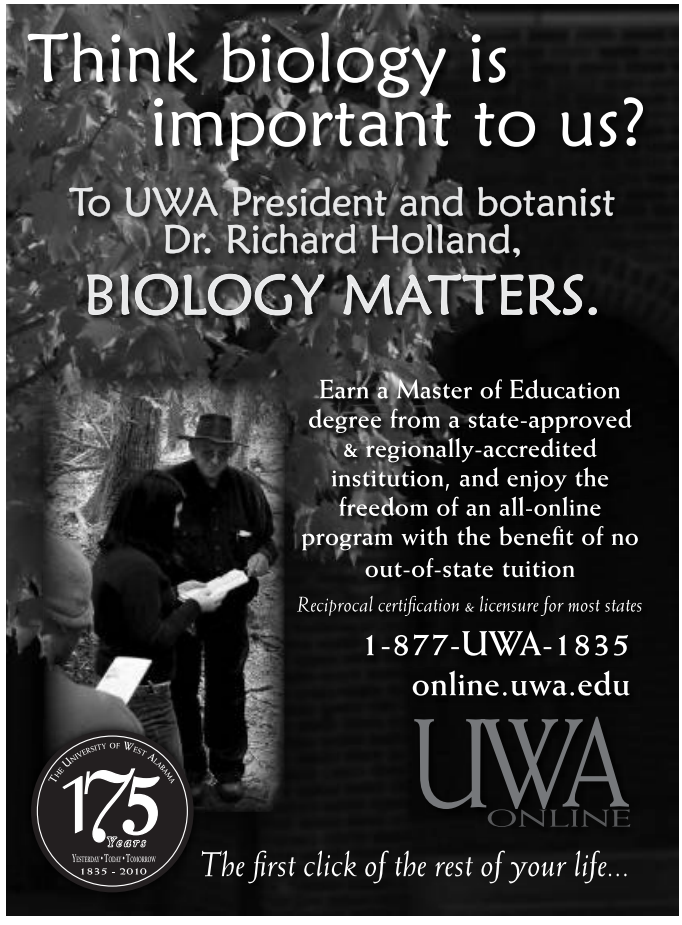

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