

An Open-Ended Investigative Microbial Ecology Laboratory for Introductory Biology

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Abstract: In this article we describe a multi-week investigative laboratory in microbial ecology/diversity and nitrogen cycling that we have used in our introductory biology course. This module encourages active student involvement in experimental design, using the scientific literature and quantitative analysis of large data sets. Students analyze soil microbial community diversity using EcoPlates (sole source carbon utilization patterns). Also, functional analysis of these communities includes assays for nitrate, nitrite and ammonium to determine experimental effects on the nitrogen cycle. Students are continually assessed by various strategies throughout the module.

Keywords: Investigative, introductory biology, quantitative analysis, microbial diversity, soil microcosms, nitrogen cycle

INTRODUCTION

Numerous reports continue to emphasize the need for reform in science curricula (Handelsman *et al.*, 2004; DeHaan, 2005). These reform efforts encourage active learning, investigative approaches and collaboration. Laboratory experiences are extremely important in learning the process of science. Here, we describe a multi-week investigative laboratory module based on microbial community ecology for an introductory college biology laboratory course (Jones-Held, *et al.*, 2004). Typically, we use this module with first year students from various science disciplines. The scheduled laboratory sessions are three hours in length.

We focused on microbial community ecology because this theme illustrates that organisms do not exist in isolation from each other and from their environment. Furthermore, the community ecology approach introduces the concept of biodiversity and its relationship to microbial communities that cannot be directly observed. Additionally, the method used for evaluating microbial diversity generates large data sets that students analyze and this helps improve and reinforce their quantitative and computer skills. We coupled the microbial community analysis to the microbial cycling of nitrogen as it illustrates the role of living organisms on chemical transformations, reinforcing the link between biology and chemistry that is so important for students at the beginning stages of their science studies. The specific learning objectives of this module are to design and initiate an experiment that will examine environmental effects on soil microbe community diversity; to understand the importance of soil microbes and their diversity in nutrient cycling within ecosystems.

Ecologists often characterize communities in terms of species diversity. Species diversity is usually determined by using diversity indices that take into account both richness, reflecting the number of species present, and evenness, indicating the relative abundance of species in a community (Molles, 2008). These indices rely on taxonomic identification that is fairly straightforward for plants and animals. Microbial diversity in the environment is much more difficult to address or quantify because taxonomic identification of naturally occurring microbes is usually inconclusive and difficult (Kirk *et al.*, 2004). However, characterizing naturally occurring microbial communities is extremely important because of their essential role in ecosystem functions like nutrient cycling and in assessing the effects of any environmental perturbations on the community. An important component to terrestrial nutrient cycling is the soil environment and its diverse microbial communities. There are existing methods for characterizing soil microbial diversity (Kirk *et al.*, 2004). One of these methods uses a commercially available 96 well plate (EcoPlate) to characterize the functional diversity of the microbial community using sole source carbon utilization patterns (Figure 1). The basis for the analysis is metabolic profiling using 31 different carbon sources and a blank. In the presence of a carbon source that bacteria can use for growth, their respiratory activity will reduce the tetrazolium dye present in each well of the plate producing a visible color change. Using these plates, microbial diversity can be quantitated using existing diversity and evenness indices that have been modified to reflect plate color development (Zak *et al.*, 1994; Staddon *et al.*, 1997; Staddon *et al.*, 1998;

Derry *et al.*, 1998). Plate color development reflects functional diversity not taxonomic diversity (Staddon *et al.*, 1998). From an instructional standpoint, these plates are straightforward to use and each EcoPlate generates a large data set that students can organize and quantitatively analyze (Jones-Held *et al.*, 2004).

The nature of the individual components of the module and their integration for successful completion provide a framework for applying tools and strategies designed to provide both formative and summative assessment of essential student skills at this level such as: (a) understanding and appreciating the biodiversity and roles within ecosystems; (b) application of the elements of the scientific method within a specific context; (c) appropriate execution of laboratory practices and use of laboratory equipment; (d) collection, analysis, interpretation and evaluation of data; (e) search, retrieve and utilize published and electronic forms of scientific information. A timetable and list of activities for this module is presented in Table 1. Each activity is subsequently described in detail.

MATERIALS AND METHODS

Pre-laboratory Preparation

Materials: Plastic Containers (27.9 cm X 44.5 cm X 17.8 cm); Commercial Potting Mix or Soil; Seeds (i.e. peas, beans, corn, grass)

Typically, this module of our introductory course is offered during the winter months when it is difficult to obtain field-collected samples. Using field-collected samples would shorten the scheduling of the module and provide the advantage of having students collect their own field samples. Instead we set up soil microcosms 4 to 6 weeks prior to the start of semester so that the plants have time to establish and grow for several weeks before the students begin their treatments. Microcosms are small self-contained ecosystems that have been used in many ecological research studies. Plants are all started at the same time by randomly sowing seeds across each microcosm. Even though all microcosms have the same potting mix we try to add some diversity to each microcosm by using different plants or combinations of plants. We maintain the microcosms under greenhouse conditions until student use. Within

the 4 to 6 week growth period under greenhouse conditions all plants are of sufficient size for students to initiate their experiments.

Laboratory Sessions 1 – 3

Materials: Microcosms; 7.6 cm diameter pots; Balances, Spatulas, Distilled water; pH Meters; Other standard laboratory equipment; glassware and chemicals as needed

The first week of lab involves an overview of the module providing the framework for students to search the literature and to develop an experimental design. Some designs have included the effects of salinity, temperature or metals such as copper on the soil microbial community. Laboratory instructors discuss with the students what they need to consider in setting up an experiment. Within this one week period, student groups (3 to 4 students/group) must search the literature and develop a written experimental design. During the previous semester our students have had two information literacy modules that provide a proper context for conducting literature searches. Alternatively, the fundamentals of literature searches could be integrated into the first laboratory session. Instructors meet with each of the groups during the second week session to discuss the scientific rationale and feasibility of the proposed experiment. Students need to be aware that they must coordinate with other group members in order to be consistent in their experimental treatments. This component introduces students to the need for collaboration in experimental research studies.

In laboratory session 3, after student groups have formalized their experimental plans, we provide each group with a microcosm of their choice. The groups are provided with all the appropriate materials according to their individual experimental protocols. Instructors are present to assist if questions or problems arise. Some of the questions that are typically encountered relate to chemical solubility and making stock solutions. Also, students are required to maintain a proper lab notebook so they know their experimental set-up and how to consistently prepare solutions over their two-week treatment periods. Students are permitted to come into the lab whenever it is open and available.

Using the microcosms as their soil source, students subdivide the microcosms into 7.6 cm

Table 1. A timetable and sequence of laboratory activities for the module.

Time	Activity
Four to six weeks prior to start of module	Set-up soil microcosms
Session 1	Literature search and developing an experimental design
Session 2	Review and discussion of experimental design
Session 3	Setting up experiments - Initiating experimental treatments
Session 4	Continued treatments of microcosms
Session 5	Prepare soil extracts, inoculate EcoPlates and cultures for nitrogen analysis
Session 6	Read EcoPlates. Complete ammonium, nitrate and nitrite analyses
Session 7	Data Analysis

diameter pots by randomly removing soil plugs. The soil plugs are removed using bulb planters and spatulas. Students have the option to include the plants with the soil plug or not. The number of pots depends on the experimental design and number of replicates. Once students establish their samples they are maintained on growth stands on a 16-hour light and 8-hour dark cycle at room temperature in the laboratory. During the treatment periods groups are responsible for maintaining their samples. Optionally students can make plant growth measurements (i.e. height) or record digital images of their samples that can be used in their presentations.

Laboratory Session 5 – Initiate EcoPlates and Samples for Nitrification

Materials: EcoPlates (Biolog Inc. 3938 Trust Way, Hayward CA 94545, USA - \$10.51/plate); Multichannel pipettor; Disposable reservoirs (Biolog Inc. 3938 Trust Way, Hayward CA 94545, USA); Microplate Reader (Tecan U. S. Inc., Research Triangle Park, N.C. 27709); Buchner funnels; Whatman #1 filter paper

Preparation of soil microbial extracts

In the preparation of the soil microbial extracts all essential glassware is autoclaved and sterile technique is emphasized as much as possible throughout the preparation of the microbial extracts and subsequent inoculations. Soil (10 g) from the experimental treatment(s) or control sample are each transferred to a separate flask containing 90 mL of sterile 0.85% NaCl (flasks are labeled appropriately). The samples are placed on a shaker at room temperature for one hour in order to release the bacteria from soil particles. After shaking, the soil in each sample is allowed to settle out before collecting the supernatant. Alternatively, if particulate soil

matter remains in suspension, the sample is filtered through a Buchner funnel with Whatman #1 filter paper or centrifuged to obtain a particulate-free filtrate or supernatant. Adjustments in the inocula densities can be made at this point if necessary. In our experiences, preparing the soil microbial extracts as described always provides sufficient inocula densities without adjustment to obtain results with the EcoPlates. As a guideline, typically, a soil microbial extract with an absorbance value of at least 0.100 at 592 nm is used.

Inoculation of EcoPlates

Soil filtrate (20 mL) from the experimental extract or control sample is transferred to a pipette reservoir. Using a multi-channel repeating pipettor, with tips, 150 µL of soil filtrate is transferred to each well of the EcoPlate. The EcoPlates are covered by lids and each plate is appropriately labeled. The plates are incubated at 28°C for 3 to 7 days (temperature and length of incubation are variables that can be part of the experiment).

Nitrogen cycle

The production of soil ammonia/ammonium from organic matter decomposition involves a variety of bacterial species as well as fungi. Depending on the environmental conditions, this released ammonium may be oxidized to nitrite that is further oxidized to nitrate. The oxidation of ammonia in soils is primarily accomplished by members of the genus *Nitrosomonas*. Nitrite oxidizers are in the genus *Nitrobacter*. The biological transformations of ammonia and nitrite comprise the process of nitrification, part of the global nitrogen cycle. In order to isolate and analyze the different microbial genera involved with ammonia production and its

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
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
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
G1 D-Cellulose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethylamine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Fig. 1 EcoPlate Carbon Sources. Each carbon source is repeated three times across the microplate. Only the first four columns of the plate are shown in this figure.

subsequent oxidation, various enrichment media for the different nitrogen transformations are used and inoculated with soil from the microcosms.

Materials: Sterile Nitrate Producing Medium (Nitrite oxidizers) in tubes (10 mL per tube; Paerl, 1998); Sterile Nitrite Producing Medium (NH₄ oxidizers) in tubes (10 mL per tube; Paerl, 1998); Sterile Peptone Broth (4% peptone, w/v) in tubes (10 mL per tube)

Culture set-up

Tubes of each type of media are obtained in replicate. To each tube 0.1 g of soil from the experimental treatment is added. This step is repeated using the control sample and another set of replicate tubes of each type of media. All tubes are appropriately labeled. The tubes are incubated at 28°C for one week (temperature and length of incubation are variables that can be part of the experiment).

Laboratory Session 6 - Complete Experimental Analyses

Materials: Test tubes (13 X 100 mm); Cuvettes; Distilled water; Balances; Weigh boats; Spatulas; Spectrophotometer or Spec 20; Pipettors; Pipette tips; Vortexer; Concentrated HCl; Incubator; Ammonium Ion-Selective Electrode (Vernier Software & Technology, 13979 SW, Millikan Way, Beaverton, OR 97005-2886 - \$179); High and Low Standard Ammonium solutions (Vernier Software & Technology); Vernier LabPro – Computer Interface; Computer; Low Range Test Kit (The Nitrate Elimination Co., Inc. 334 Hecla St. Lake, Linden, MI 49945 - \$40.00 for 25 assays)

Analyses

EcoPlate: After one week, sample absorbances are read at 592 nm using a microplate reader. Results are printed out.

Ammonium Determination: Students measure ammonium using an ammonium electrode interfaced with a computer. Direct measurements for ammonium can be made using the calibrated electrode according to the manufacturer's instructions. There are ammonium electrodes commercially available that connect to a pH meter providing an alternative for ammonium determination.

Nitrite and Nitrate Assays: Nitrite and nitrate assays are performed according to the described protocol by the supplier. The assay is based on the enzyme nitrate reductase, catalyzing the reduction of nitrate to nitrite using the electron donor NADH. The nitrite reacts with color reagents under acidic conditions to produce a visible color. For both assays,

25 µL from each of the sample tubes are routinely used. Assay buffer is added to each sample and standard. Then a supplied NADH source that is appropriately diluted is added to each tube and vortexed. Nitrate reductase is added to each tube (samples and standards). The tubes are incubated at room temperature for 20 minutes. Subsequently, the supplied color reagents are added and mixed with the samples and standards and incubated at room temperature for 10 minutes. The absorbances of all the standards and samples are read at 540 and recorded after appropriately blanking the spectrophotometer. These values are used for nitrate determination in the samples. The assay for nitrite follows the same steps as described for nitrate except nitrate reductase and NADH are omitted. After completion of the assay, a standard curve (using the supplied and appropriately diluted nitrate standard) is constructed and the concentration of nitrite and nitrate in each of the samples are determined. For the nitrate determinations it is essential to include samples that only contain the nitrate producing medium since this medium contains nitrite which is the end product determined in both assays.

RESULTS AND DISCUSSION

As this is an introductory biology course, we developed specific guideline questions for students because they are at the early stages of learning how to analyze and interpret data. The following questions and experimental analyses are addressed and included in student laboratory papers and presentations. We encourage student groups to think of alternative ways to analyze data or other questions that could be addressed.

Initially, the students do find the task of transforming the raw data ultimately into summary diversity and evenness indices daunting. Additionally, students need the basic background information on diversity indices and evenness and what these parameters indicate about a community. Usually we devote an entire laboratory period to explain how to organize the raw data for subsequent analysis. This process involves using a sample set of data and going through the different steps of data transformation as described in Tables 2 and 3. Also, at this time we further explain the concept of diversity indices. We have included examples of actual EcoPlate data from a student group experiment and the subsequent data transformations (Figure 2;

Table 2. Calculations for microbial community analysis (from Derry *et al.*, 1998).

Parameter	Equation	Variable
Shannon Diversity Index	$H' = -\sum p_i \ln p_i$	p_i = proportional color development of the i^{th} well over total color development of all wells of a plate
Shannon Evenness	$E = H'/\ln S$	S = number of wells with color development

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.477	2.787	2.106	2.057	0.330	2.791	2.263	2.230	0.307	2.716	2.306	2.365
B	2.248	2.953	2.790	2.510	2.115	3.047	2.522	2.711	2.097	2.799	2.596	2.395
C	1.956	1.640	0.801	1.365	2.108	1.812	0.698	1.345	2.267	1.708	0.445	1.472
D	1.951	3.165	1.461	2.475	1.832	3.048	0.943	2.453	2.063	2.969	1.526	2.376
E	2.489	2.438	1.941	1.356	2.805	2.830	1.771	1.487	2.506	2.841	1.918	0.988
F	2.535	1.237	1.712	1.876	2.762	1.783	1.500	1.467	2.512	1.726	1.335	1.395
G	2.949	1.809	1.522	2.435	2.808	1.889	0.921	2.623	2.936	1.765	1.524	2.478
H	2.337	0.755	2.084	1.937	2.400	0.728	2.161	1.460	2.325	0.576	0.502	1.471

Fig. 2. A sample result of a microplate scan of an EcoPlate. The absorbances were derived from a student experiment examining the effects of salinity on the soil microbial community. The soil extract used to inoculate this plate originated from a soil microcosm treated with 50 mM NaCl for two weeks. The EcoPlate was incubated at 28°C for one week. Each absorbance value reflects microbial growth on a specific carbon source and a blank (n=3).

Tables 2 and 3).

Questions posed to the students

Microbial Community Analysis.

- For the analysis of your plates refer to Table 2. Complete the calculations for microbial diversity and evenness as described in Table 2. Since the EcoPlate wells are in triplicate, calculate the Mean Diversity Index and standard deviation. Calculate the mean Evenness value and standard deviation.
- Explain the results of EcoPlate analyses.
- What other possible effects may have contributed to the results you found with the EcoPlates?
- What other experimental variables may affect the interpretation of the data generated by EcoPlates?
- What may be possible limitations to using EcoPlates in the analysis of microbial communities?
- Was there any correlation between nitrification and microbial diversity?

Nitrogen Cycle

- Was the extent of ammonification the same in all the samples? If not, what factors could account for the differences between samples? Explain.
- Was the extent of nitrification the same in all samples? If not, what factors could account for the differences between samples? Explain.
- Summarize and present your nitrification data in an effective manner.
- Using the appropriate scientific literature resources, find information on whether your experimental treatments (i.e. salt, temperature, pH) have been found to have an effect on elements of nitrification or the Nitrogen Cycle.

Student assessment

Student assessment consists of the following components:

Lab Conduct and Demeanor: The first semester of our laboratory schedule is largely devoted to learning techniques and proper lab protocols for

Table 3. Sample calculations for diversity and evenness based on one set of replicates from Figure 2. These calculations are based on the absorbance values in cells A1 through H4 of Figure 2. A spreadsheet (i.e. Excel) or an appropriate statistical application (i.e. Minitab) is used for data calculations and reduction.

Sequence	Description of Calculations
Step 1	Subtract blank (Cell A1 – Figure 2) from all absorbance values (A2 through H4).
Step 2	After subtracting blank, divide each absorbance value by the sum of all the absorbance values of a set of replicates. In this example, sum all the absorbance values from A2 through H4 and divide each absorbance value by this sum. This calculation will give the (p_i) for each well or cell.
Step 3	Take the (\ln) of each (p_i) and multiply by the (p_i). Sum all of these values and take the negative of this sum. In this example, that value is 3.353 and is the calculated H' (diversity index) for that set of replicates.
Step 4	Repeat Steps 1-3 for the other two replicate sets (i.e. A5 to H8 and A9 to H12).
Step 5	Determine the Mean \pm S.D of the replicates (Table 4). Repeat calculations using other plates in experiment. Use an appropriate statistical test for the analysis of sample data (i.e. unpaired t-test).
Step 6	The evenness is calculated by taking each H' and dividing by natural log of the number of wells showing color development. For example, with the first set of replicates (Figure 2) $S = 31$. The \ln of 31 = 3.43. $E = 3.353/3.43 = 0.98$. Repeat calculations for the other sets of replicates and plates. As with the diversity index, determine the Mean \pm S.D of the replicates (Table 4). Use an appropriate statistical test for the analysis of sample data (i.e. unpaired t-test).

Table 4. Means and standard deviations (n = 3) for Shannon Diversity Indices and Evenness. This transformed data is based on raw data (**Figure 2**) generated by a student group examining the effects of salinity on microbial community diversity. Experimental Group A – soil samples were treated with 50 mM NaCl for two weeks. Experimental Group B – soil samples were incrementally subjected to increasing NaCl to a final concentration of 140 mM NaCl over two weeks. The student group used soil microcosms and protocols described in this paper.

Treatment	Shannon Diversity Index	Shannon Evenness
Control (distilled water)	3.236 ± 0.041	0.95 ± 0.010
Experimental Group A (50 mM NaCl)	3.341 ± 0.017**	0.977 ± 0.006**
Experimental Group B (Increasing to 140 mM NaCl)	3.311 ± 0.007**	0.967 ± 0.006

**significantly different from the Control at $p \leq 0.05$ using an unpaired t-test

gathering data, determining statistical relevance and presenting data. Lab exercises such as micropipetting, spectrophotometry, preparation and use of standard curves and Minitab applications, etc. provide a sound basis for further laboratory exercises and experiments. An important part of the laboratory is the emphasis on proper attitude and behaviors required for effective and efficient operation of a working scientific laboratory. Students are made aware that they will be evaluated on these characteristics as well as appropriate “results” of laboratory work. The projects conducted by the student groups described here allow the instructors to assess the ability of the students to apply what should have been learned during the first semester to the specific applications of their project. Observations made by instructors during the laboratory projects, specifically, errors of omission or commission relative to procedures, techniques, use of equipment, etc. are communicated to students as negative evaluations to be included in grading. These evaluations are conveyed in individual meetings with students and represent 10% of their laboratory grade.

Lab Notebooks: At the beginning of the semester, each laboratory instructor provides the students in each lab section with a handout, *Guidelines for Laboratory Notebooks*, which provides detailed description of the types of notebooks allowed for use, the manner in which entries of different types can be made, and examples from lab notebooks from past semesters. These descriptions provide the students with the assessment criteria by which their notebooks will periodically be evaluated. Routine evaluations are made at mid-semester and at the conclusion of the course; some instructors choose to evaluate the notebooks more frequently.

Written Reports: As part of the requirement for the course, a lab report, documenting the procedures and results of the experiment(s) is completed by each student group. Again, each instructor provides the students in their section(s) with a handout(s) describing the elements that will be required for a

written report. Typically, these descriptions provide explanations and examples of the usual components of a scientific lab report in the biological sciences including Introduction, Materials and Methods, Results (including construction of Tables and Figures) and Discussion. The descriptions (and examples) again provide the student with the assessment criteria that will be used for evaluation of their written reports. Most instructors provide anonymous past reports for student scrutinies which are separated into “Poor”, “Acceptable” and “Good” categories to provide the current students with examples of various applications of the assessment criteria. Additionally, most of the students have taken or are taking concurrently courses in Critical Thinking and Effective Writing. These Core Courses are part of their liberal arts education requirement. Instructors make a conscious effort to relate the scientific method components that are the core of the biology courses the students are taking to the critical thinking skills they are accumulating in the liberal arts course. Instructors also try to relate specific instructions given to students in the Effective Writing course which apply to “descriptive writing”, “argumentation”, etc. to the instructions given related to writing scientific reports in the lab sections as a way to demonstrate transferability of learning and application of assessment criteria to similar situations.

Oral Reports: Each student group completing a project is asked to present an oral report, of 15-20 minutes, using Power Point presentation techniques, to the other students in the lab section and instructor (other students and instructors are also invited to attend). Each instructor conducts a “mini-workshop” on Power Point presentations for those students who might not be familiar enough with the process. Each instructor provides specific guidelines for all aspects of the presentation which serve as the assessment criteria for this component of the lab requirement. Prior to the oral presentation, each student group must submit a hard copy of the proposed Power Point presentation for instructor evaluation; the instructor

may then suggest changes to be made before final presentation. Some lab instructors utilize peer evaluation of the Power Point presentations, using a score sheet that reflects the guidelines given by the instructor to the students which serve as the assessment criteria. Students are encouraged to “rehearse” their Power Point presentations within their group and/or other groups; instructors are also available to provide evaluations of the “rehearsals”.

For many of our students this is the first time they have been involved in a multi-week investigative laboratory activity. They find the experience stimulating and challenging. We have found this module to be very effective in actively engaging students in the process of science as well as refining their analytical and quantitative skills.

The lab is open ended with the intention of encouraging revisions, modifications and new additions to the labs. For example, after the first time using this module, we decided to include a component on serial dilutions, a common microbiological technique but one where students easily become confused. The serial dilutions were easily incorporated into the sequence at the time the students prepared their soil microbial extracts. Alternatively, this sequence of investigative analysis could be extended to include an analysis of 16S rDNA by isolating and culturing a single bacterial isolate from the community (Ros *et al.*, 2008).

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