Fire vs. Metal: A Laboratory Study Demonstrating Microbial Responses to Soil Disturbances

Mary E. Stromberger*

ABSTRACT

Incubation studies are traditionally used in soil microbiology laboratory classes to demonstrate microbial respiration and N mineralization–immobilization processes. Sometimes these exercises are done to calculate a N balance in N fertilizer-amended soils. However, examining microbial responses to environmental perturbations would appeal to soil microbiology students with broader interests in ecology and environmental soil science. In this study, perturbations of fire and heavy metal inputs were used to demonstrate disturbance impacts on microbial respiration and N mineralization, soil β-glucosidase activity, and abundance of soil bacteria and fungi in a series of exercises lasting 28 days. Students constructed duplicate microcosms containing pristine forest soil or forest soil burned by fires varying in severity or polluted with $CuCl_2$. During a 2-week period, students measured changes in soil pH, CO_2 evolution by the NaOH-trap method, and extractable soil NH_4–N and NO_3–N content. After 2 weeks, students tested for β-glucosidase activity and preserved samples for enumeration of total bacteria and fungi. Direct counts of bacteria and fungi were conducted on the third week by epifluorescence and phase contrast microscopy, but alternative methods to assess microbial biomass can be employed. Respiration was determined on the fourth week by titration of NaOH vials. After completion of the exercises, students had an appreciation of microbial responses to different types of disturbances, as measured by multiple biological parameters. Because this study was repeated over 2 years, students in the second year were able to assess soil microbial recovery immediately and 1 year after a forest fire. This study could easily be adapted for other types of disturbance issues, including salinity, land application of waste materials, and contamination of soil by xenobiotics.

LABORATORY COURSES in soil microbiology strive to instruct students in basic and sometimes advanced techniques used in soil microbiology research, and these exercises should be designed so that students have the opportunity to apply these techniques in a challenging and interesting situation. A common laboratory exercise conducted in soil microbiology courses is the incubation study, in which soils are incubated aerobically following an amendment of some sort. Students typically measure microbial activity, in the form of CO_2 evolution and/or N mineralization, over a 1- to 2-week period. Originally, the laboratory incubation study described here was designed to assess the N cycling activities of microorganisms in soils amended with organic materials or inorganic N fertilizer. This appealed to students with an agronomic background because of their interests in plant nutrition and the degradation of organic materials differing in C/N ratios. However, the majority of students now enrolled in SC456, the Soil Microbiology Laboratory course at Colorado State University, have nonagronomic majors, including civil engineering, rangeland ecology, biology, and forest science. To satisfy the interests of these students, there was a need to design laboratory exercises involving native (e.g., grassland or forest) rather than cultivated soil, with experimental treatments relevant to students' scientific background. As a result, a laboratory incubation study was designed so that students could examine microbial abundance and activities in a forest soil perturbed by a fire as well as pollution by a heavy metal, Cu^{2+}.

Forest fires have been shown to increase the total N content in burned soil, especially for the NH_4–N fraction (Prieto-Fernandez et al., 1993), and limited studies on microbial impacts indicate that fires negatively affect activities of acid phosphatase and β-glucosidase enzymes in soil (Boerner and Brinkman, 2003), fungal propagules and hyphal lengths (Vázquez et al., 1993), and cellulase-producing bacteria but not ammonifying microorganisms (Acea and Carballas, 1996). Such results indicated that a simple laboratory incubation study could reveal interesting and important microbial effects of fire to students enrolled in a soil microbiology laboratory course. Moreover, students enrolled in SC456 at Colorado State University had a sincere interest in understanding fire impacts on forest ecosystems because of a recent fire event in the state of Colorado. In June 2002, the Hayman fire burned more than 52,000 hectares (130,000 acres) of Colorado's Pike National Forest, covering parts of Park, Jefferson, Douglas, and Teller counties. This fire was particularly significant because it was Colorado's largest wildfire in recorded history, and in one particularly devastating run, it burned nearly 24,000 hectares (60,000 acres) and ran for 27 kilometers (17 miles) in 1 day (U.S. Forest Service, 2004). In addition, fuel moistures were the lowest in recorded history, adding to the unpredictability of the fire. The fire was finally contained on 2 July 2002, after the destruction of 133 residences, one commercial building, and 466 outbuildings. The final economic impact of the fire has yet to be realized, but suppression costs alone were $39 million (www.fs.fed.us/r2/psicc/hayres; verified 14 Dec. 2004).

As instructor of SC456, my objective was to develop an incubation study that would interest students of diverse academic backgrounds. Specifically, my goal was to train students in multiple techniques so that disturbance impacts on microorganisms would be gauged on multiple levels. For students, their objective was to use these techniques to assess the impacts of fire severity on soil microorganisms 1 month or 1 year after the Hayman fire. In addition, students in the second year of the study also examined the microbial response to a different type of perturbation—that of heavy metal pollution—to determine which stress (fire or Cu) was more detrimental to forest soil microorganisms.

Department of Soil and Crop Sciences, Colorado State Univ., Fort Collins, CO 80523. Received 27 Apr. 2004. Article. *Corresponding author (Mary.Stromberger@ColoState.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: PNG, ρ-nitrophenyl β-D-glucopyranoside; DI, distilled water; SALG, Student Assessment of Learning Gains.

Table 1. Equipment and supplies needed to set up one incubation study for each group of two or three students.

Quantity	Description
Microcosms	
8	500-mL mason jars with lids
150 g	soil (control or stressed)
Soil pH	
2	small beakers
2	glass stirring rods
Extractable NH_4–N and NO_3–N	
2	plastic specimen cups with lids, approximately 100-mL volume
300 mL	KCl, 2.0 *M*
1	graduated cylinder, at least 50-mL volume
6	filter papers
2	filter funnels
6	plastic scintillation vials with caps for collecting filtrate
Alkaline CO_2 traps	
6	glass scintillation vials with caps
90 mL	standardized NaOH, 0.5 *M* (exact molarity known)
1	25-mL volumetric pipet

MATERIALS AND METHODS

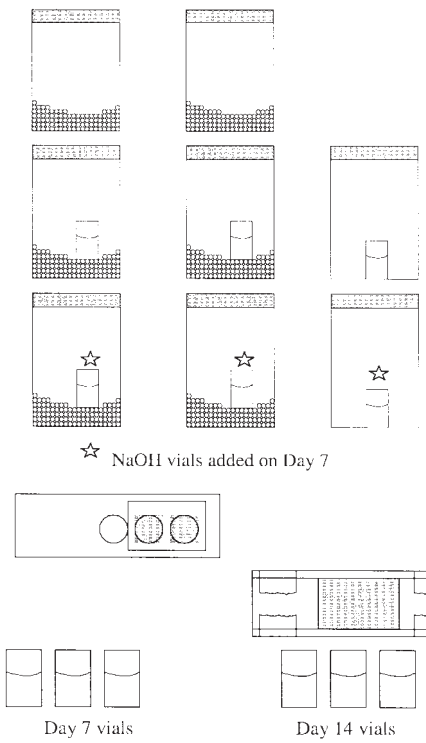
This laboratory exercise was designed for a senior-level undergraduate laboratory course in soil microbiology, which met once a week for a 3-hour period. The series of exercises below spanned a 28-day period and coincided with lectures on soil microbial growth, enzymes, and C and N biogeochemical cycles given in the lecture-based soil microbiology course. This incubation study was designed to accommodate up to eight students (two students per group, with three to four groups total), but more students can be accommodated by increasing the number of students per group or increasing the number of groups with the addition of a new treatment variable. An alternative method for microbial biomass determination, rather than direct enumerations as described below, should be conducted when there are more than eight students.

Laboratory Preparation

A complete list of material and supplies is shown in Table 1, and an overview of the laboratory exercises is given in Table 2. On the first day of the incubation study (Day 0), each group of students prepared six microcosms by adding 25 g (dry weight) soil into each of six 473-mL mason jars. Wide-mouth jars are preferred for easy insertion and removal of NaOH-filled vials (see below). In 2002, the first year this incubation study was performed, three groups of students were assigned to one of three soil treatments: pristine forest soil, forest soil 1 month after a moderate-severity fire event (defined as combustion of surface litter material and scorching of soil to a depth of ~2 cm), and forest soil 1 month after a high-severity fire event (complete vegetation and tree kill and scorching of soil to a depth of ~5 cm). In 2003, there were four student groups, and each group was assigned to one of four treatments: pristine forest soil, forest soil 1 year after a moderate-severity fire or high-severity fire, and pristine forest soil affected by an input of $CuCl_2$. Each microcosm was labeled with the group number and/or treatment. Microcosms were randomly divided into three duplicate sets, and each set was labeled with a sampling day (e.g., Day 0, 7, or 14) and replicate designation (e.g., A or B). Students then adjusted the moisture content of their soil to 75% of the moisture content at –1/10 bar by adding 5 mL of distilled (DI) water or 5 mL of 0.1 *M* $CuCl_2$ (for Group 4 of 2003) dropwise to each microcosm. Because Cu is extremely toxic, lower concentrations of $CuCl_2$ could be added to soils while still causing changes to microbial popu-

Table 2. Time schedule of procedures to complete for measuring microbial biomass and activity in a laboratory incubation study.

Sampling date	Procedures to complete
Day 0	1. Soil pH 2. Extractable NH_4–N and NO_3–N
Day 7	1. Remove and cap NaOH vials from Day 7 jars; add NaOH vials to Day 14 jars 2. Soil pH 3. Extractable NH_4–N and NO_3–N
Day 14	1. Remove and cap NaOH vials from Day 14 jars 2. Remove and freeze soil subsample for direct counts of bacteria and fungi 3. Soil pH 4. Extractable NH_4–N and NO_3–N 5. β-Glucosidase enzyme assay
Day 21	1. Prepare bacterial mounts for direct enumeration of total bacteria; count bacteria using epifluorescence microscopy when time permits 2. Prepare slides and measure lengths of total fungal hyphae by phase contrast microscopy
Day 28	1. CO_2 evolution determination by titration of NaOH vials



lation dynamics and activities. For example, a more realistic $CuCl_2$ amendment rate (e.g., 200 ppm) could be employed, depending on the objective of the instructor and/or students. After the amendment, each group obtained two additional mason jars, which were labeled Day 7 Blank and Day 14 Blank. To complete the microcosms, each group of students added 15.0 mL of standardized NaOH (0.5 *M*) to each of three glass scintillation vials; these vials were placed into each of the Day 7 jars (two jars with soil and one blank). The exact concentration of the NaOH used was recorded in laboratory notebooks. Finally, the jars were capped securely and placed in a 25°C incubator. Before the laboratory, the soils had been sieved through a 5-mm mesh screen, and the moisture contents had been determined. Also, the pH meter was calibrated and solutions of 2 *M* KCl, standardized NaOH, and 0.1 *M* $CuCL_2$ were prepared before the Day 0 exercise.

On Day 7, the next laboratory meeting periods, students carefully removed the NaOH vials from their Day 7 microcosms. Tongs were used to remove vials if students' hands were too large to fit inside the jars. The vials were then capped and stored in the freezer for later analysis. To complete the preparation, 15.0 mL of standardized NaOH were added to each of three new glass scintillation vials, and these vials were added to Day 14 jars, which were then returned to the 25°C incubator.

Laboratory Analyses

After preparing their microcosms, students were required to determine the pH values of Day 0 soils and extract Day 0 soils for inorganic N analysis. For each of the two jars labeled Day 0, groups hand-mixed the soil to aid in homogenization and weighed out 10 g dry weight soil into beakers for pH determination. Students added 10 mL of DI water to each beaker, and each slurry was stirred intermittently for 30 minutes. The pH of each soil slurry was determined by a pH electrode and meter. Students then weighed an additional 10 g dry weight soil from each microcosm into a plastic specimen cup. Soils were extracted in 50 mL of 2.0 *M* KCl for 30 minutes on a shaker set at 240 rpm. Students filtered the extract through filter paper (Whatman no. 2) into plastic scintillation vials, and the vials were capped and stored in the freezer for later inorganic N analysis. Soil pH determination and extraction for inorganic N were repeated on Day 7 and 14 of the incubation with duplicate microcosms labeled Day 7 and Day 14, respectively. After the Day 14 laboratory period, the instructor delivered all KCl extracts (including blanks) from Year 1 to the Colorado State University Soil, Water, and Plant Testing Laboratory for analysis of NH_4–N and NO_3–N. In Year 2, KCl extracts were analyzed in the laboratory of Dr. Dwayne Westfall and Ms. Lucretia Sherrod using an Alpkem autoanalyzer (OI Analytical, College Station, TX).

After soils were analyzed for pH and extracted for inorganic N on Day 14, groups divided the remaining soil into subsamples for additional analyses. Approximately 5 g of soil were removed from each Day 14 microcosm, placed in a Ziploc freezer bag, and stored in the freezer for later determinations of the relative abundances of soil bacteria and fungi. Of the remaining soil, 1.0 g (dry weight) was removed from each microcosm and assayed for β-glucosidase enzyme activity as described by Tabatabai (1994). Alternatively, another soil enzyme could be examined, such as arylsulfatase or acid or alkaline phosphatase. Enzyme assays typically require little time and are relatively simple to perform. For these laboratory incubation studies, student groups began the enzyme assay first, and during the 1-hour incubation time, groups measured the pH of their soil samples and extracted soils for inorganic N. Before the Day 14 laboratory, standard solutions of known concentrations of ρ-nitrophenol were prepared as described by Tabatabai (1994). Each group then read the absorbance (410 nm) of the standards on the spectrophotometer, and each student generated his or her own standard curve using a software tool such as Excel.

On Day 21, students prepared microscope slides for total bacterial enumerations as described in Table 3. Students also prepared fungal slides from soil suspensions and measured lengths of fungal hyphae in soil (Table 4). Before laboratory exercises on Day 21, dilution tubes containing sterile DI water, SYBR I reagent, antifade solution, and a small flask of molten agar (kept in a water bath set to 45°C) were prepared. Each student made one bacterial and one fungal slide by se-

Table 3. Procedures for direct enumeration of total bacteria. Adapted from Bloem et al. (1995) and Weinbauer et al. (1998).

Description

Materials and supplies

1. Dilution tubes containing sterile distilled (DI) water
2. Shaker
3. 1-mL pipets
4. 10-µL pipettor and tips
5. Reich counting slides (Bellco Glass, Vineland, NJ)
6. Bunsen burner or other flame source
7. Plastic tray with cover
8. Staining baths with slide holders for rinsing
9. Stain solution consisting of 1 µL green fluorescent dye SYBR I (Bio-Rad Laboratories, Hercules, CA) mL^{-1} TE buffer (10 m*M* Tris-HCl, 1 *M* EDTA; pH 7.5). CAUTION: avoid skin contact and inhalation!
10. Antifade solution [50% glycerol, 50% phosphate-buffered saline (130 m*M* sodium chloride, 10 m*M* sodium phosphate buffer, pH 7.5), 0.5% ascorbic acid]
11. Coverslips
12. Low-fluorescence immersion oil
13. Clear nail varnish
14. Epifluorescence microscope equipped with an ocular grid and a filter set for blue light (BP 450–490 nm exciter filter)

Procedures

1. Add 1.0 g (dry weight) soil to a screw-capped test tube containing 9.5 mL of sterile DI water.
2. Shake tube on shaker for 10 minutes at 240 rpm (high setting).
3. Prepare a 1:100 dilution. Shake the 1:10 dilution tube for 30 seconds, let the tube sit for 30 seconds, and then remove a 1.0-mL aliquot from the tube. Transfer the aliquot to a test tube containing 9.0 mL of sterile DI water.
3. Shake the 1:100 dilution tube, and transfer 10 µL of the suspension to the center of a marked circle on the microscope slide. Repeat for a second, adjacent marked circle.
4. Smear the suspension to cover each of the two 1-cm^2 areas. Air dry the smears and heat-fix by passing through a flame.
5. Place slides on moistened paper towels in a plastic container. Flood each smears with 50 µL of SYBR I stain for 15 min in the dark. Cover the trays during staining to prevent slides from drying.
6. Gently blot dry the slides with Kimwipes and air dry.
7. After air-drying, mount a cover slip over the slide with a small drop of antifade solution. Seal the edges with nail varnish. At this point, the slides can be stored at 2°C until observation.
8. Perform counts using an epifluorescence microscope at 1000× magnification. Count 10 fields of view per circle.
9. For each circle, calculate bacterial numbers per gram of soil as follows:

$$\text{Bacteria per gram soil} = (N/X)\,(A/B)\,(1/S)$$

where:
N = the total number of bacteria counted in one circle
X = the number of fields of view counted in one circle
A = area of the slide covered by the sample (1 cm^2)
B = area of the field of view (i.e., area of ocular grid) *To be provided by instructor*
S = amount of soil smeared on the slide (0.1 mg or 0.0001 g)

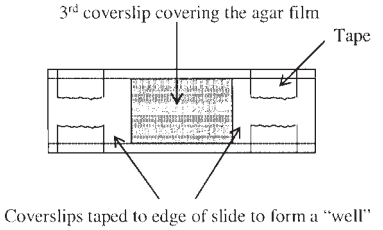
Table 4. Procedures for direct enumeration of total fungi. Adapted from Lodge and Ingham (1991) and Olson (1950).

Description

Supplies

1. Dilution tubes containing sterile distilled (DI) water
2. Shaker
3. 1-mL pipets
4. Molten agar solution, 1.5%
5. Water bath, ~45°C
6. Coverslip well slides (2 per person)

To make, tape two coverslips of known thickness to a microscope slide, aligning the edge of each coverslip to the edge of the microscope slide. Make sure the ends of the tape do not overlap.



Procedures

1. Add 1.0 g (dry weight) soil to a dilution tube containing 9.5 mL of DI water.
2. Shake tube on shaker for 10 minutes at 240 rpm (high setting).
3. Prepare a 1:100 dilution. Shake the 1:10 dilution tube for 30 seconds, let the tube sit for 30 seconds, and then remove a 1.0-mL aliquot from the tube. Transfer the aliquot to a test tube containing 9.0 mL of sterile DI water.
3. Shake the 1:100 dilution tube, and transfer 1.0 mL of suspension to a clean test tube. Add 1.0 mL of molten agar to the same tube and mix the contents gently. (Note: this represents a final dilution of 1:200).
4. Add the soil solution/agar suspension dropwise to the area of the microscope slide between the two coverslips. Make sure the agar covers the area completely.
5. Press a third coverslip down onto the agar to produce an agar film of known thickness.
6. Observe fungal hyphae at 400× by phase contrast microscopy.
7. Using the ocular grid, count the number of lines intersected by the fungal hyphae, excluding the very top and far left lines.
8. Count a total of 15 fields (example, three transects of five fields each).
9. Calculate meters of fungal hyphae per gram of dry soil as follows:

$$\text{Meters hyphae per gram soil} = (N/X)\,(W/V)\,(D)\,(\pi/4)$$

where:
N = the total number of lines intersected by fungal hyphae
X = the total number of fields counted
W = the width of one ocular grid line (m)
V = the field volume (mL; area of ocular grid × thickness of one coverslip)
D = the dilution factor (200)
$\pi/4$ = conversion factor derived from equations of Olson (1950)

Table 5. Titration method for CO_2 evolution. Adapted from Fuhrmann (1994) and Zibilske (1994).

Description

Introduction

Sodium hydroxide reacts with carbon dioxide as follows:

$$CO_2 + 2\ NaOH \longrightarrow Na_2CO_3 + H_2O$$

Carbonate ions can be removed by forming barium carbonate:

$$Na_2CO_3 + BaCl_2 \longrightarrow BaCO_{3(pt)} + NaCl$$

Any remaining (unreacted) NaOH can then be measured by titration with a strong acid such as HCl. The difference between the amount of NaOH initially present and the amount remaining following incubation can be used to calculate the amount of CO_2 evolved as follows:

$$\text{mg } CO_2 = (\text{m.e. base} - \text{m.e. acid})(22)$$

where:
m.e. base is the milliequivalents of NaOH originally present
m.e. acid is the milliequivalents of HCl required to titrate the excess base
22 is the milliequivalent weight of CO_2
Note: milliequivalent = volume (mL) × N (milliequivent/ mL)

Materials

1. Standardized HCl, ca. 0.5 *M*
2. 50-mL burets (1 per group)
3. buret stands and clamps (1 per group)
4. $BaCl_2$, 0.5 *M*
5. 250-mL Erlenmeyer flasks (6 per group)
6. 50-mL graduated cylinder (1 per group)
7. Phenolphthalein solution (1 g phenolphthalein in 95% ethanol)
8. Dilute HCl for rinsing glassware

Procedures

1. If not already done, quantitatively transfer samples to individual 250-mL flasks with the aid of several distilled water rinses.
2. Using a pipet or graduated cylinder, add 15 mL of 0.5 *M* $BaCl_2$ to each flask. A white precipitate of $BaCO_3$ should form.
3. Add five drops of phenolphthalein indicator to the contents of a flask and immediately titrate, while swirling the suspension, with standardized HCl to a white end-point. Add two more drops of indicator to check the end-point (the suspension should remain white). If you over-shoot the end point, add a measured volume of standard NaOH and retitrate (be sure to record the volume of additional NaOH).
4. Record the exact volume and molarity of the HCl used in the titration.
5. Discard the suspension, rinse the flask with dilute HCl (do not use standardized acid) to remove traces of $BaCO_3$, and thoroughly rinse again with tap followed by distilled water.
6. Calculate the rate of CO_2 evolution per gram (dry weight) of soil after subtraction of CO_2 from the blank samples.

lecting one of their group's Day 14 soils that had been stored in the freezer. Wearing gloves, each student prepared and stained two soil smears (two adjacent circles) so that each student had two analytical replicates of one soil sample to enumerate for total bacteria. Once prepared, slides were labeled with the student's name, placed in a slide storage box, and stored in the refrigerator. Students scheduled appointments with the instructor to receive one-on-one instruction on use of the epifluorescence microscope, which was used to visualize green-fluorescing bacterial cells against a dark background under high (oil immersion) magnification.

Using the same soil sample, each student also prepared one agar film slide for measuring total fungal hyphal lengths as described in Table 4. To more efficiently organize Day 21 exercises, students were divided into two groups so that some prepared the fungal slides while others prepared the bacterial slides; this divided students so that not everyone was ready to use the microscope for fungal measurements at the same time. Once slides were prepared, students were individually instructed on using the phase-contrast microscope, the counting of fungal hyphae that intersected lines of the ocular grid, and capturing of images using the digital camera and software system. The instructor also demonstrated the need to focus up and down through the agar film to view fungal hyphae through the depth of the film. Students printed an image of fungal hyphae from their slide to paste into their notebook. Because of time constraints, the number of fields counted was restricted to 15, but the need to count more fields to reduce variability was discussed. Generally, once an agar film slide was prepared, a student could count his or her slide within 10 minutes. However, students prepared their agar film slides in stages, because once a slide was made, it needed to be examined shortly before the agar film dried and cracked. While waiting a short time (10–20 minutes) to be examined, slides were kept moist between damp paper towels.

On Day 28, the last day in the series of incubation study exercises, groups determined the amount of CO_2 evolved from Day 0 to 7 and Day 7 to 14 during aerobic incubation. Vials of NaOH were titrated with standardized HCl after addition of $BaCl_2$ and phenolphthalein indicator as described in Table 5. All reagents (standardized HCl, $BaCl_2$, and phenolphthalein) were prepared before the beginning of this laboratory exercise.

Laboratory Lecture

On the first day of the incubation study, students were given a brief introduction on the Hayman fire event, followed by a discussion of some forest fire impacts on soil microorganisms. Published journal articles that described forest fire effects on microbial populations and activities were distributed to each student to aid in future discussions (Prieto-Fernandez et al., 1993; Vázquez et al., 1993; Acea and Carballas, 1996). In addition, students in 2003 were given a summary of the laboratory results from 2002, which described the immediate effects of the forest fire on soil microorganisms. Students were then presented with an overview of the exercises to be completed during the 28-day period (Table 2). Before setting up the microcosms on Day 0, students were instructed in the proper use and storage of the pH electrode and meter.

On Day 7, students compared the Day 0 soil pH results and noted any differences among soil treatments. Groups in 2003 were able to discuss how soil pH values 1 year after the fire compared with pH values in 2002. In addition, students were instructed that the NaOH-filled vials from Day 7 jars should, under ideal conditions, be titrated immediately. Instead, the Day 7 vials would be stored in a freezer and CO_2 evolution would not be determined until all the vials were collected. This simplified the laboratory exercises by allowing all vials to be titrated in one laboratory period.

On Day 14, students were given an overview of a general soil enzyme assay, which emphasized that soils are incubated under ideal conditions to maximize enzyme activity. Students were reminded that the enzyme β-glucosidase catalyzes the breakdown of cellobiose into glucose monomers, and they were instructed that ρ-nitrophenyl β-D-glucopyranoside (PNG) would be added to soil to serve as the target substrate. The reaction of PNG with soil β-glucosidase to produce glucose and ρ-nitrophenol, a yellow chromaphore, was also discussed. A demonstration was given on the proper use of a spectrophotometer to measure absorbance values of filtered samples. Students were instructed on how the enzyme activity in each of their soil samples would be quantified based on a standard curve, which each student would create using absorbance values of solutions with known concentrations of ρ-nitrophenol. After students completed the exercise for Day 14 (described below), groups presented their soil pH data for Day 0, 7, and 14, and a brief discussion was held regarding treatment effects on soil pH over time.

Day 21 of the incubation study began with a brief discussion of results from the enzyme activity assay. Afterward, an overview was given to students on different methods for assessing microbial biomass in soils, including direct enumeration by microscopy and indirect measurements by the chloroform-fumigation incubation and chloroform-fumigation extraction techniques. The discussion described some advantages and disadvantages of direct enumeration techniques, such as the ability to differentiate between bacterial and fungal abundances (advantage) but that measurements can be tedious, cause eye-strain, and require the counting of many fields of view to reduce variability (disadvantages). The use of various staining reagents, such as DAPI, SYBR I, and fluorescein diacetate, was also discussed. Finally, students were given a chalk-board demonstration on how to assess fungal hyphal lengths using the grid-line intersect method as described by Olson (1950). Students were given a copy of this paper (Olson, 1950) for their records.

On the last day of the incubation study, Day 28, students were instructed on the procedures for CO_2 evolution determination based on titration of NaOH vials with standardized HCl. The calculations to determine milligrams CO_2 respired from Day 0 to 7, and Day 7 to 14 were reviewed. The difference between normality and molarity was also discussed. Finally, students summarized the effects of fire severity and $CuCl_2$ on relative abundances of soil bacteria and fungi, microbial C and N mineralization activity, and β-glucosidase enzyme activity.

Laboratory Assignment

Students were required to maintain a laboratory notebook describing all exercises in the laboratory course. Notebooks included a table of contents and a brief description of the objectives, materials, and supplies for each exercise. All data were recorded in the notebook, and results of each exercise were summarized and discussed in paragraph form. Also included in the notebook were printouts of any software-generated data analysis (e.g., the linear regression equation for the ρ-nitrophenol standard curve) and photographic images of bacterial cells and fungal hyphae. Calculations to determine the number of bacterial cells, meters of fungal hyphae, milligrams CO_2 evolved, and rate of enzyme activity on a per gram dry soil basis also were shown in the laboratory notebook. In addition, at the completion of the incubation study students were required to write an overall summary that discussed the effects of forest fire severity and heavy metal inputs on soil microbial growth and activity.

RESULTS AND DISCUSSION

By the end of the 28-day laboratory study, all students had acquired practical experience in the measurement and interpretation of microbial abundance and activities in pristine and perturbed soils. Overall, students had little difficulty in completing the exercises as long as they had read the exercise procedures (detailed in their laboratory manual) before each laboratory period. For some students, generating the ρ-nitrophenol standard curve required individual instruction, mainly because they were not familiar with a particular software program.

The most time-consuming exercise was the direct enumerations of total bacteria and fungi because only one phase contrast/epifluorescence microscope was available. For this course, enumeration of bacterial slides required individual instruction and time outside of the scheduled laboratory period, although this was not a large problem because each student generally needed only 15 to 20 minutes to receive instruction and count his or her slide. Direct enumeration of fungal hyphae must be completed by all students within the laboratory's allotted time period, however, because the agar film slides cannot be stored after they are prepared. Therefore, direct enumeration exercises for total fungi are recommended only for laboratory classes of small size (<10). In addition to being time-consuming, direct enumerations required special equipment, including a phase contrast/epifluorescent microscope fitted with an ocular grid. Although a digital camera and imaging software is not necessary, students in this course particu-

Table 6. Summary of data generated by student groups for the first 2 years of the laboratory incubation study. Groups were assigned to a different forest soil treatment: control, soil burned by moderate-severity fire, soil burned by high-severity fire, and for Year 2, soil polluted with 0.1 *M* $CuCl_2$.

Measurement	Control	Moderate severity fire	High severity fire	$CuCl_2$
		Year 1		
pH Day 0	5.8	6.2	6.9	
pH Day 7	5.7	6.3	7.5	
pH Day 14	5.4	6.4	7.6	
		mg L^{-1}		
NH_4–N Day 0	0.90	8.65	5.90	
NH_4–N Day 7	3.45	19.5	12.9	
NH_4–N Day 14	2.35	23.4	15.5	
NO_3–N Day 0	0.40	0.40	0.30	
NO_3–N Day 7	4.50	0.90	0.40	
NO_3–N Day 14	7.05	1.75	0.20	
		μg CO_2 g^{-1} day^{-1}		
Respiration Day 7	52.6	342	229	
Respiration Day 14	43.4	50.6	91.3	
		μg ρ-nitrophenol g^{-1} h^{-1}		
β-glucosidase	126	58.6	28.2	
		cells g^{-1} dry soil		
Total bacteria	1.35×10^8	6.75×10^7	3.49×10^8	
		m hyphae g^{-1} dry soil		
Total fungi	252	290	137	
		Year 2		
pH Day 0	7.0	6.5	7.1	4.6
pH Day 7	5.9	6.0	6.5	4.6
pH Day 14	5.7	6.1	6.5	4.8
		mg L^{-1}		
NH_4–N Day 0	1.68	2.68	12.3	0.89
NH_4–N Day 7	0.75	5.53	17.1	16.0
NH_4–N Day 14	1.05	4.79	11.5	21.8
NO_3–N Day 0	2.77	17.7	21.2	2.19
NO_3–N Day 7	9.24	24.0	22.0	4.07
NO_3–N Day 14	14.6	14.6	21.3	1.98
		μg CO_2 g^{-1} day^{-1}		
Respiration Day 7	100	−16.3	97.3	87.4
Respiration Day 14	72.0	13.5	88.1	74.6
		μg ρ-nitrophenol g^{-1} h^{-1}		
β-glucosidase	154	46.4	43.3	210
		cells g^{-1} dry soil		
Total bacteria	1.03×10^9	5.55×10^8	2.07×10^8	4.42×10^8
		m hyphae g^{-1} dry soil		
Total fungi	97.4	34.4	207	188

larly enjoyed being able to capture images of bacterial cells and fungal hyphae from slides they had prepared. Images were stored on a computer and printed so that they could be pasted into students' laboratory notebooks. The benefits of direct enumeration exercises were that (i) students learned a classical method for estimating fungal and bacterial abundance that also involved state-of-the-art technology (i.e., digital camera and imaging software), and (ii) students were able to visually observe bacterial cells and fungal hyphae in soil, an opportunity quickly disappearing from microbiology laboratories as scientists come to depend on biochemical and molecular-based techniques to assess the status of microorganisms in soil.

Due to the constraints of direct enumerations for a laboratory class, microbial biomass can be assessed alternatively by the chloroform-fumigation extraction procedure if the instructor has access to a dissolved organic C analyzer. The chloroform-fumigation incubation method could also be done, although it would involve additional NaOH titrations following the 10-day incubation period. Bacterial and fungal numbers could also be determined by plate count methods. This was previously done for burned and nonburned soil in this course, where students plated diluted soil suspensions onto plates containing 0.1-strength tryptic soy agar or Martin's rose bengal agar to enumerate bacteria and fungi, respectively, by the spread plate method. However, this exercise was conducted in the second week of the laboratory course rather than at the end of this incubation study.

A key part of each laboratory period of the incubation study was the discussion of results gathered so far. At the beginning of every laboratory period, students wrote on the board their results from the previous week's exercise. Results were summarized in a table that was distributed to the students as new information was added (Table 6). This stimulated discussion among the class regarding fire and heavy metal effects on microbial activity and abundance. Students observed that 1 month after the Hayman fire, nitrification activity appeared to be depressed in burned soil compared with control soil, as indicated by the lack of NO_3–N formation and no decline in soil pH. Relatively high levels of NH_4–N in burned soil were attributed by some students to mineralization of organic N during combustion of soil organic matter. Respiration activity was higher in burned soil, whereas β-glucosidase activity was reduced in burned soil relative to the nonburned control. The abundance of fungal hyphae was also reduced in soil 1 month after being burned by the high-severity fire.

One year after the fire, students observed that some soil properties seemed to have recovered from the forest fire. For example, levels of NH_4–N in soil burned by moderate-severity fire were similar to those found in control soil. A relatively high amount of NO_3–N, with a decline in soil pH during the first week of the incubation study, indicated that nitrification was occurring in burned soil. However, net nitrification (i.e., change in NO_3–N from Day 0 to 14) was still greater in control soil relative to burned soil 1 year after the fire. Soil respiration activity was similar among all treatments, although experimental errors occurred in Year 2 for the group assigned to the moderate severity fire treatment. This led to a discussion of possible explanations for low CO_2 evolution measured, including loosely capped microcosms or titration errors. One year after the fire, students noted that β-glucosidase activity in soil remained depressed, but the abundance of fungal hyphae in soil burned by the high-severity fire was much greater than that in the control soil. Some students speculated that because of lower bacterial abundances in the high-severity fire treatment, competition among microorganisms was reduced, which allowed for fungal proliferation in this treatment.

Finally, in Year 2, students compared the relative impacts of forest fire with heavy metal pollution due to a $CuCl_2$ amendment. Most students noted that $CuCl_2$ addition resulted in dramatic reduction in soil pH and NO_3–N levels but increased β-glucosidase enzyme activity fivefold compared with soil burned by the high-severity fire. Low soil pH values were attributed to the acidity of the $CuCl_2$ solution (pH = 4.0), and students associated low nitrification activity and high fungal abundances in $CuCl_2$-perturbed soil with the decrease in soil

pH. An increase in β-glucosidase activity was more difficult to explain, although one student hypothesized that activity increased because Cu^{2+} was being used as a cofactor for enzyme activity. Overall, most students concluded that the $CuCl_2$ perturbation had greater detrimental effects on microbial communities than fire 1 year after the Hayman fire, as evidenced by the decrease in soil pH and low nitrification activity. One student concluded that the $CuCl_2$ amendment may have killed or inhibited the development of many microbial species, thus reducing microbial diversity as a whole, whereas microorganisms resistant to Cu^{2+}, including fungi, flourished in the environment due to reduced competition.

This incubation study can be modified to allow for additional exercises. For example, students have pooled the remaining soil from Day 14 jars and used it in a later exercise to enumerate denitrifying bacteria by a most-probable-number technique. This exercise demonstrated that denitrifier numbers were very low (<200 cells g^{-1} soil) in $CuCl_2$-amended soil. Moreover, different soil perturbations can be applied, depending on the interests of students. If soil burned by a forest fire is not available, soils can be heated for 1 hour at a high temperature (e.g., 220 or 460°C) in a muffle furnace to simulate burning by a fire (Giovannini et al., 1990). Or soils can be perturbed by addition of one of many possible amendments. For example, salt solutions could be added to mimic salt marsh soils or to compare nonsaline vs. saline agricultural soils. Pharmaceutical compounds such as antibiotics could be added to mimic potential environmental contamination through land application of animal manures. Diesel fuel or xenobiotics could also be added so that students could assess impacts of fuel spills or synthetic pesticides, for example, on soil microorganisms. Ideally, students should discuss and choose early in the course the types of perturbations and develop hypotheses to test so that the incubation study can be modified to their interests.

Laboratory exercises should by dynamic in nature and evolve over time to accommodate students' needs as well as to make improvements. For example, this particular set of laboratory exercises can be strengthened in future years by incorporating a statistical analysis of the data set. Students could pool their data and run analysis of variance tests on soil microbiological and chemical parameters to increase the analytical skills learned as well as to enhance the exercise in terms of data interpretation and experimental design. Also, an assessment component would be a valuable addition that could gauge, in an unbiased way, the effectiveness of the laboratory in achieving instructor and students' goals. For example, within 1 week of completing the laboratory, students could complete an on-line survey of Student Assessment of Learning Gains (SALG) (Seymour, 1997) to assess their satisfaction of the teaching exercises. The SALG is a free survey instrument whose context can be modified by instructors with different learning objectives, and it has been used, for example, to assess different tools for teaching soil erosion concepts in an introductory soil science course (Mamo et al., 2004).

CONCLUSIONS

This series of laboratory exercises provided a practical study of disturbance impacts on soil microbial abundance and activity in a forest soil. Students were able to learn multiple microbiological techniques in an incubation study that provided insight to potential ecosystem-level impacts of forest fires and soil pollution by heavy metals. This created an engaging learning situation because students generally had a sincere interest to understand the impacts of the Hayman fire event, Colorado's largest wildfire in recorded history, as well as the impacts of copper pollution that may occur as a result of acid mine drainage. Importantly, the treatments chosen for this type of study can be selected based on student interests, thereby empowering students to design their own experiments and test their own hypotheses.

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Soils may become contaminated by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition [1, 2]. Heavy metals constitute an ill-defined group of inorganic chemical.Â are oxidized to carbon (IV) oxide by microbial action, most metalsÂ Recent studies on some New Zealand soils treated with biosolids have shown increased concentrations of Cd, Ni, and Zn in drainage leachates [33, 34]. 2.4. Wastewater.