INQUIRY & INVESTIGATION

Using Grass Germination to Measure the Toxicity of Household Substances & Teach Statistical Methods

NABT BioClub RECOMMENDATION

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ABSTRACT

We describe a series of three experiments in which students develop a model system for measuring the LC50 of household substances, using grass seed as the model organism. Students use statistical methods to compare two samples (using chi-square and Student's t-tests), conduct a two-level multifactor experiment to look at multiple factors simultaneously and observe interactions, and make serial dilutions to measure the LC50 over a threefold concentration range. The experimental series was very inexpensive to run and tended to provide very successful LC50 measurements.

Key Words: *grass germination; toxicity; LC50; lethal concentration; statistical methods.*

○ Introduction

LC50 and LD50 (median lethal concentration and median lethal dose, respectively, for 50% of a population) are commonly reported toxicology measures of substances. LC50 is typically used for concentrations of gaseous substances that are lethal to 50% of a population and can also be used for the concentration lethal to 50% of aquatic species; LD50 is typically used for solid and liquid substances and is usually reported as mg/kg of body weight. Note that *population* refers to all members of a given set of individuals, a sample from which is tested to determine the LC50 or LD50 of that population. Classroom experiments to measure LC50 for lettuce seed, duckweed, and *Daphnia* have been developed by the Environmental Inquiry program at Cornell University (see "Bioassays," http://ei.cornell.edu/toxicology/bioassays/index.html).

To teach these concepts in an introductory-level college environmental science course, the lead author developed a four-week series of experiments in which students measure the LC50 for germinating grass seed. In the first two weeks, students examine environmental factors that affect grass-seed germination in order to find conditions to maximize germination rate, such as soil type, grass-seed variety, amount and duration of lighting, and so on. Using the optimal conditions discovered in the first two weeks, the students perform a final germination experiment in which serial dilutions of a household substance are added to the soil.

The series was inspired by what the lead author felt were practical parts of the scientific method for students to learn, based on his previous experience as an engineer, especially with the use of statistical methods (useful references for statistical methods include Box et al., 1978; Sokal & Rohlf, 1981; Zar, 2010). Because the class was developed for a popular general education elective, it was stressed that even non-science majors are likely to encounter statistics in a future career. The series has evolved over a 10-year period, with both authors having taught it, and the success rate of the experiments is very high. The statistical analyses are done using Microsoft Excel for ease of accessibility; however, more advanced software packages could be used, such as SPSS Statistics for Windows.

The first part of the series teaches students how to develop appropriate experimental models and the need to have proper controls in place (factors that are kept constant) in order to make meaningful conclusions. Before beginning the LC50 experiment, the students must understand that certain environmental conditions promote a high rate of grass germination, so that they know that any lethal effects can be attributed to the action of the household substance. In the case of grass-seed germination, it is easy to come up with many easily manipulated factors, such as type of grass seed, depth of planted seed, amount/frequency of water, type/duration of light,

> *"This four-week-long lab exercise is an excellent way to demonstrate how scientists develop an experimental design to examine certain phenomena."*

Table 1. Idealized results of a two-factor ANOVA experiment measuring the mass of plants.

No Interaction		
Amount of Light	**No Fertilizer Mass**	**Fertilizer Mass**
12 hours	9 g	10 g
24 hours	11 g	12 g
Interaction Present		
Amount of Light	**No Fertilizer Mass**	**Fertilizer Mass**
12 hours	11 g	10 g
24 hours	9 g	12 g

drainage/no drainage, humidity (covered/uncovered), and so on. Students are assigned or allowed to pick a single factor to test at two values in a classical one-factor experiment. Besides the values chosen for the factors, the instructor emphasizes that the values of the controlled factors (constants) are just as important as the values of the factors that are being varied.

The series teaches three main statistical techniques: Student's *t*-test to compare the means of two populations, a chi-square test to compare two frequencies, and a multiple-factor experiment to compare means of multiple populations using analysis of variance (ANOVA). In both Student's *t*-test and chi-square test, the result is a *p*-value (*p* standing for probability). Understanding the meaning of *p*-values is a very important learning objective. The multiple-factor experiment typically challenges the students' understanding, since much of their exposure to experimentation in high school has been limited to a single-factor experiment. The results of the multiple-factor experiment allow students to see which experimental factors have the most effect on the measurements obtained in the experiment, to note any interactions between factors, and to screen for optimal germination conditions.

In the final step of the series, the optimal germination conditions are used to germinate grass in soil to which serial dilutions of a household substance have been added. The students are required to make a graph of the germination rate (linear scale) vs. substance concentration (logarithmic scale). The logarithmic scale is needed to expand out the low concentrations since the experiment looks at a range covering four orders of magnitude. From the graph students determine both an approximate value for the LC50 and a statement regarding their sense of the precision based on the amount of variability among samples with identical concentrations.

The overall series of experiments is an excellent way to teach scientific inquiry. The material supply costs for the experiments are minimal, especially with the reuse of the planting trays from year to year. The major learning objectives are as follows. Upon completion of the LC50 experiment series, the student will

(1) understand the importance of controlled factors in designing one-factor experiments;

(2) understand the meaning of a *p*-value, and that it can be calculated for both comparing the mean value of two populations and comparing a proportion between two populations;

(3) understand that experiments can be done in which more than one factor is varied, and that such an experimental design can help discover interactions among factors; and

(4) be able to set up an experiment with serial dilutions of a household product and use the data to estimate an LC50 value as well as a sense of the precision of the estimate.

○ Background on Statistical Techniques

A starting point for discussing statistical techniques with students is to ask whether they can make any conclusions about two populations by comparing only one sample from each. An example would be to have a blond-haired student and a dark-haired student stand up and compare heights; the students are then asked if they can use these data to make any conclusions about whether blond-haired people have a different average height than dark-haired people. After they say no, they are asked how someone would determine whether there was a difference in average heights of blondes and dark-haired people. The student response is typically that you need to measure a lot of people, but only rarely would a student know how to analyze the data once they were collected. This leads to the first week's experiment, which looks at the effect of a single factor and is evaluated by both a Student's *t*-test and a chi-square test.

The concept of statistical hypothesis testing is briefly introduced, explaining that the null hypothesis is that there is not a statistically significant difference between two populations and that the alternative hypothesis is that there is a difference (type 1 and type 2 errors are typically not discussed, to avoid complexity at this introductory level).

Student's *t*-test is used to compare the mean (average) values of samples from two populations. The result of the test is a *p*-value, which can be described as the probability that one would find an equal or greater difference between the means if the two samples were drawn from identical populations.

The chi-square test is used to compare frequencies in two or more populations – for example, the frequencies of deaths following various medical treatments for a given disease. The result is again referred to as a *p*-value, with a similar but slightly different meaning: the probability that one would find an equal or greater difference between the *frequencies* if the samples were drawn from identical populations.

The second week's experiment looks at multiple factors simultaneously in one experiment using a two-level, multifactor ANOVA design (note that *levels* could refer to different categories if not quantitative values). Table 1, showing idealized results of two-factor experiments in which the effects of interactions can be seen, is used to introduce the concepts to the students. In the case of "no interaction," the observation is that fertilizer resulted in a 1 g increase in mass regardless of the amount of light, just as 24 hours of light resulted in a 2 g increase in mass regardless of whether the fertilizer was used. When an interaction is present, the students can see that whether the fertilizer is beneficial depends on the amount of light, just as whether the 24 hours of light is beneficial depends on the fertilization. It is pointed out to the students that even

Table 2. Multifactor setup.

Factor	High Level	Low Level
A. _____		
B. _____		
C. _____		
D. _____		

Table 3. Multifactor sample numbering.

Samples	Factor A	Factor B	Factor C	Factor D
1	High	High	High	High
2	High	High	High	Low
3	High	High	Low	High
4	High	High	Low	Low
5	High	Low	High	High
6	High	Low	High	Low
7	High	Low	Low	High
8	High	Low	Low	Low
9	Low	High	High	High
10	Low	High	High	Low
11	Low	High	Low	High
12	Low	High	Low	Low
13	Low	Low	High	High
14	Low	Low	High	Low
15	Low	Low	Low	High
16	Low	Low	Low	Low

though the example looks at only two levels, more than two levels could be used for an ANOVA design. For example, there could be three or more light levels or fertilizer levels. It is also noted that in order to allow statistical testing of the ANOVA results, replication of each set of levels is necessary, just as replicates are needed to perform a *t*-test.

Previous results of a four-factor experiment are also shared, using a spreadsheet to show what the effect of each factor is along with the interactions between factors. For simplicity, only a single experimental setup is done and the students compare the relative sizes of the effects and interactions. If desired, multiple setups could be done and a statistics package such as SPSS could be used to get an *F*-value, the test statistic that measures the significance of each factor and interaction of an ANOVA design in a similar manner as a *p*-value measures a difference in means in one-factor experiments. Note that the experimental design is complex, as it involves examination of the effects of each of four factors and of four two-way interactions, and could also examine six three-way interactions and a four-way interaction (the latter two are not discussed with students, due to the complexity).
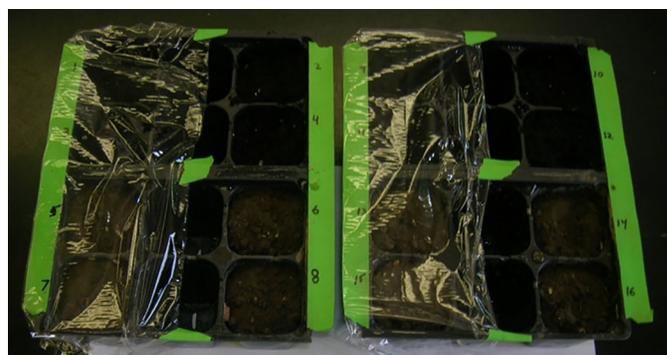
○ Experimental Procedures

In Week 1, students work individually to design their own unique experiments to measure grass-seed germination by varying one factor. Factors that could be examined include type of soil, type of grass seed, planting depth, frequency of watering, amount of water, covered or uncovered with plastic wrap, duration of light (light cabinets were provided in the lab), and artificial vs. natural lighting. The students must choose the values of the factor to be varied as well as values for all the factors that remain constant. Students are given two six-cell seed-germinating trays (1.5 inches square by 2.25 inches deep) and are instructed to plant exactly 10 seeds in each cell under the conditions chosen for both the experimental factor (one factor for each tray) and the controlled factors. One week after the start of the experiment, the students count how many seeds germinated and measure the height of a random sampling of 15 of the seedlings from each tray. The students analyze their data using an online spreadsheet set up for both a chi-square test for number germinated and a *t*-test for seedling height comparisons.

In Week 2, after collecting data from the first experiment, the students work in groups of three or four to perform a multifactor experiment to find optimal conditions for grass-seed germination. The students should draw on their experience from the first experiment to pick "high/low" levels for four factors that they want to study and fill in Table 2, and then use these values as shown in

Sample Multifactor Experiment
Factor A: Window location (south vs. north)
Factor B: Soil Type (potting vs. loam)
Factor C: Seed size (large vs. small)
Factor D: Covering (present vs. absent)

Figure 1. Multifactor experiment setup. Note that Factor A was chosen to be window location (south or north), which allows cells 1–8 to be in a separate location from cells 9–16 (cell numbers to sides of cells in photo).

Table 3 to set up 16 different sample-germinating conditions. Four six-cell seed trays are used, with the middle two cells left empty. The students are tasked with determining a logical setup (such as that shown in Figure 1). They plant exactly 20 seeds in each cell. At the end of one week, they count the number of seeds that germinated in each cell and enter the values in a spreadsheet to

Table 4. Multifactorial example results. Negative values mean that the "low" level had more germinated seeds than the "high" level. Since no replicates of the experiment were performed, we cannot determine F-value, but it is apparent that seed size and covering as well as the interaction between those two factors had large impacts on germination.

Sample	Number Germinated			Analysis – all numbers refer to number of seeds germinated			
				Factor Levels (High vs. Low)	Mean of High Level	Mean of Low Level	Difference
1	15						
2	0		**Factor A**	South vs. north exposure	4.75	5.38	−0.63
3	2		**Factor B**	Potting soil vs. loam soil	5.13	5.00	0.13
4	0		**Factor C**	Big vs. small seeds	8.50	1.63	6.88
5	17		**Factor D**	Covered vs. uncovered	10.00	0.13	9.88
6	0						
7	3		**Interactions**				
8	1		A × B	−1.13			
9	18		A × C	−0.38			
10	0		A × D	−0.88			
11	6		B × C	−0.63			
12	0		B × D	0.38			
13	18		C × D	7.13			
14	0						
15	1						
16	0						
Grand mean	5.06 seeds germinated						

**Figure 2.** A typical LC50 experiment; the LC50 in this case was between 2% and 4%.

determine the effect of each factor as well as that of each possible two-factor interaction. Table 4 shows an example output.

In Week 3, the student groups set up an LC50 experiment using the set of conditions that resulted in the highest germination rate in the multifactor experiment. It works best to use as the "toxins" solid household substances in granular form, such as table salt, detergents, and fertilizers (too much fertilizer can prove toxic). The substances should be ground to powder if they are large grains or pellets. Liquids have also been used, but they tend to be messy and hard to mix with the soil. Twelve different serial dilutions of the toxin are created in two sets, as shown in Figure 2. Visual aids are available online to help explain how to make the serial dilutions (see the Google Drive link below, under Additional Information). After making the serial dilutions and adding them to the soil, the students plant 20 grass seeds in each cell. After one week, they count the number germinated in each toxin concentration. The students are taught how to plot the LC50 graphs using Microsoft Excel, which includes using a logarithmic x-axis (needed to expand low concentrations on the graph) and error bars representing the standard deviation for each concentration (typical results are shown in Figure 3).
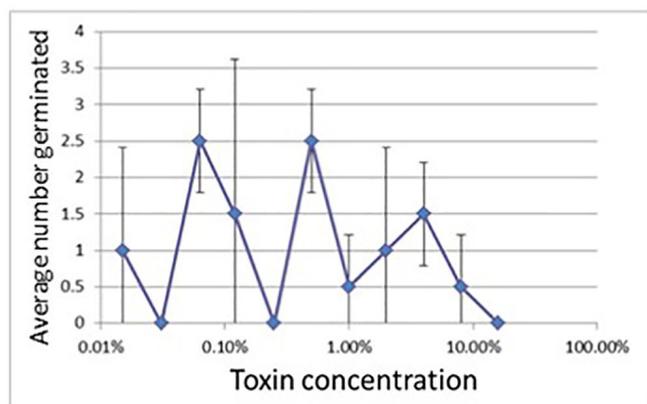
○ Assessment

For the one-factor individual experiments, students write up a full lab report. As part of the lab report, the students need to write in statistical language the null hypothesis, alternative hypothesis, and
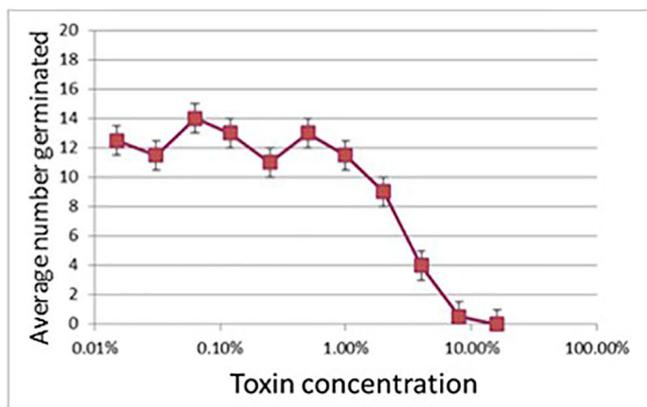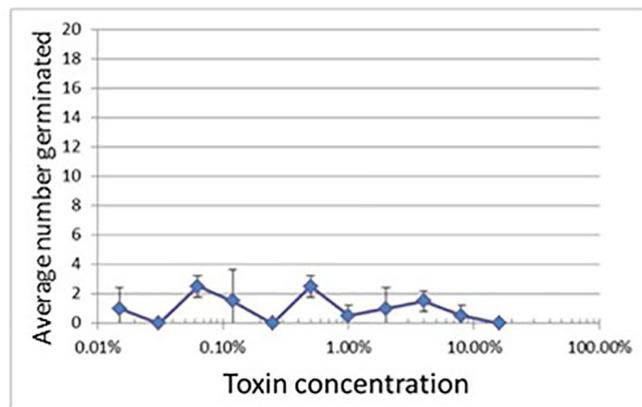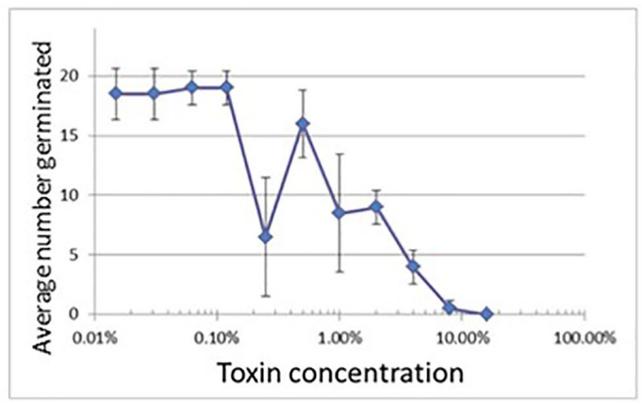
Figure 3. LC50 graphical results. Instruction for making graphs using Microsoft Excel are available on Google Drive (see link in text, under Additional Information).

acceptance criteria for determining whether there is a statistically significant difference between the two levels of the factor that was tested. The students are instructed to write the methods portion with enough detail that someone could repeat the experiment exactly the way they did it. A detailed rubric is provided to the students (available online) that guides them on the proper writing of the lab report.

The multifactor experimental results are usually reported in a worksheet format to rank the factors and interactions in order of the magnitude of their effects and to briefly summarize how they used the data to choose the conditions for the LC50 experiment. The LC50 results are also written in a full lab report form. The LC50 graph is the basis for the students' conclusions, in which they need to estimate the LC50 if possible, and state how precise they are in the estimate based on the variability they see with the error bars.

○ Conclusions

This four-week-long lab exercise is an excellent way to demonstrate how scientists develop an experimental design to examine certain phenomena, in this case the LC50 of a household substance. The one-factor test is helpful to show the difference between comparing frequencies vs. comparing means, which can be a very confusing concept in the introduction of statistics. The multifactor experiment teaches students that it is not necessary to examine only one factor at a time when doing experiments, and in fact, multifactor experiments can be used to look at interactions, which is not possible in a one-factor experiment (note that replicates are needed for determining the significance of interactions). Finally, the students should be able to develop an optimized method for grass-seed germination that will allow for toxicity measurements, in which the only impacting factor should be the substance being testing. This curriculum has been used in general education college courses and in high school classes taught by Marian University education graduates who took the college course and requested the curriculum.

○ Additional Information

For teachers interested in implementing this curriculum unit, all materials and detailed instructions can be found on Google Drive at https://drive.google.com/drive/folders/0B4hGjeH2y43XVkY0a EdOMlN0dW8.

The following Science and Engineering Practices of the *Next Generation Science Standards* (NGSS Lead States, 2013) are addressed in these experiments:

2. Developing and using models

3. Planning and carrying out investigations

4. Analyzing and interpreting data

8. Obtaining, evaluating, and communicating information

References

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