Bioassay Protocols

Protocol 1. Serial Dilutions

Objective: To make a serial dilution for use in dose/response bioassays.

The idea behind a bioassay is that the test organism will respond in a predictable way to varying concentrations of a chemical compound. You can test this idea using dose/response experiments (e.g., Protocol 2). For example, if you treat lettuce seeds with concentrated NaCl solution, they will not germinate. Treated with water containing no salt, most of the seeds should germinate. At intermediate salt concentrations, the number of seeds that germinate and the amount they grow should vary depending on the solution concentrations.

The first step in carrying out a dose/response experiment is to create a wide range of concentrations of your test solution. Initially, you probably will have no idea what concentrations will kill your test organisms and what concentrations will cause them no harm. Therefore it is a good idea to start by testing a very wide range of concentrations. You can do this by creating a serial dilution – a series of solutions, each of which is ten times more dilute than the one from which it is made.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Percent Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 g/l = 25,000 mg/l = 25 parts per thousand</td>
<td>100%</td>
</tr>
<tr>
<td>2.5 g/l = 2,500 mg/l = 2.5 parts per thousand</td>
<td>10%</td>
</tr>
<tr>
<td>0.25 g/l = 250 mg/l = 250 parts per million</td>
<td>1%</td>
</tr>
<tr>
<td>0.025 g/l = 25 mg/l = 25 parts per million</td>
<td>0.1%</td>
</tr>
<tr>
<td>0.0025 g/l = 2.5 mg/l = 2.5 parts per million</td>
<td>0.01%</td>
</tr>
<tr>
<td>0.00025 g/l = 0.25 mg/l = 250 parts per billion</td>
<td>0.001%</td>
</tr>
</tbody>
</table>

As you can see, each solution is one-tenth as concentrated as the one just above it in Table 1. The solution in the bottom row of the table has a concentration measured in terms of parts per billion (ppb). This may seem inconceivably small and impossible to measure, but in fact biological organisms are sensitive to many different chemicals in concentrations as low as parts per billion. Many drinking water standards are measured in this range. For example, the drinking water standard for lead is 15 ppb, meaning that public water suppliers must take action if concentrations higher than this are measured in their drinking water supplies.

You may be wondering what “100% concentration” means. It simply means the highest concentration in your dilution series. It can be whatever concentration you choose – just remember to write down what chemical you are using and what concentration (in mg/l) you have selected to be the 100% concentration (see Step 1).
Materials (per group except where indicated)
- Balance (can be shared by class)
- 6 beakers, flasks, or plastic cups (100-ml or larger)
- 1 100-ml graduated cylinder
- 1 10-ml graduated cylinder or graduated pipette
- Tape or wax pencil for labeling flasks
- Parafilm or plastic wrap (unless Protocol 2 is begun immediately)
- Test chemical (consult with your teacher)
- 2 L distilled water or spring water

Procedure

1. Label the 6 beakers with the percent concentrations listed in Table 1, adding the name or chemical formula for your test chemical. For example, your label might read “NaCl 100%”. Make sure to record the concentration of your 100% solution:
   \[100\% \text{ solution} = \text{____ mg/l of } \quad \text{(chemical name)}\]

2. Consult your teacher to determine the amount of your particular test chemical to use. Weigh it out and place it in the beaker labeled “100%”. Add 100 ml water and swirl gently until the chemical is fully dissolved.

3. Using a pipette or graduated cylinder, transfer 10 ml of your 100% solution to the beaker labeled “10%”. Add 90 ml of water and swirl gently to mix. Caution: Never pipette by mouth.

4. After thoroughly rinsing the pipette, transfer 10 ml of your 10% solution to the beaker labeled “1%”, then add 90 ml water and swirl to mix.

5. Continue with this dilution process until you have made all five dilutions. Unless you will be using the solutions right away, cover them tightly with plastic wrap to prevent water loss through evaporation.
Protocol 2. Dose/Response Experiments using Lettuce Seeds

Objective: To conduct a dose/response bioassay using lettuce seeds.

Background:
A bioassay is an experiment that uses living things to test the toxicity of chemicals. One kind of bioassay is a dose/response experiment, in which you expose the test organisms to various doses of a chemical and then measure their responses. In this protocol, lettuce seeds are the test organisms. After placing lettuce seeds in dishes containing various concentrations of a chemical, you count how many seeds have sprouted and then measure the lengths of the roots that have grown.

For example, if you place lettuce seeds in Petri dishes containing a concentrated copper sulfate solution, none of the seeds will sprout. If you place seeds in dishes containing distilled water, most of them should germinate and grow. At concentrations in between, the number of seeds that sprout and the length of their roots should vary depending on the concentrations of the solutions.

In this protocol, you will carry out a dose/response experiment to test the sensitivity of lettuce seeds to the serial dilutions you created in Protocol 1.

Materials (for each student or group)
- 105 Buttercrunch lettuce seeds
- 21 100 mm Petri dishes and plastic bag(s) to hold them
- 21 round paper filters (7.5 or 9 cm diameter)
- 50 ml 10% bleach solution (5 ml household bleach in 45 ml water)
- Tape or wax pencil for labeling the Petri dishes
- Metric rulers graduated in mm
- Tweezers
- 1 small transfer pipette with attached bulb (e.g., 5 ml graduated)
- Funnel and coffee filters (for rinsing seeds)
- 6 ml of each of the chemical solutions made in Protocol 1
- 6 ml distilled water or spring water from the source used in Protocol 1

Procedure
1. Soak the lettuce seeds in a 10% bleach solution for 5 minutes, and then rinse thoroughly. This kills bacteria and fungi that can interfere with seed germination.

2. In each of 21 9-cm Petri dishes, place a 7.5-cm paper filter. Label 3 dishes with the name and concentration of each of the solutions you made in Protocol 1. Label the last 3 dishes “control.”
3. To each Petri dish, add 2 ml of the appropriate test solution. Thoroughly rinse the pipette between solutions. *Caution: Never pipette by mouth! Instead, be sure to use a pipette bulb or a syringe-style pipette to avoid accidentally getting a mouthful of your chemical solution.*

4. In the control dishes, use distilled or spring water as your test solution, depending on which type of water you used to make your serial dilutions in Protocol 1.

5. To each dish, add 5 pretreated lettuce seeds, spaced evenly on the filter paper so that they do not touch each other or the sides of the dish.

6. Place the dishes in a plastic bag and seal it to retain moisture. Incubate the seeds in the dark for 5 days (ideally at a constant temperature of 24.5°C).

7. At the end of the 5-day growth period, count and record how many seeds in each dish have germinated. For each sprout, measure the radicle length to the nearest mm. *(The radicle is the embryonic root).* Look carefully at the plants to make sure you are measuring just the radicle, not the shoot as well. For example, in the picture below, you would measure just the part between the two arrows, not the rest of the sprout to the left. Enter your data in Tables 2a and 2b.

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Measure Here
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8. Graph the mean (average) for each treatment using Figures 2a and 2b. Then analyze your data using the guidelines below.

**Analysis – How Good Are Your Data?**

Once you have counted how many seeds sprouted, and measured their radicle lengths, then what? How can you interpret these results?

**Comparison to the Control**

The first thing to check is your control (the dishes that contain water rather than a chemical solution). The purpose of the control is to identify how well the seeds will grow without any added chemicals. Would you expect all the seeds in your control dishes to germinate? Probably not, just like a gardener does not expect all the seeds in a garden to sprout.
If fewer than 80% of the seeds in your control dishes sprouted, something may have gone wrong in your experiment. Perhaps the seeds were too old or stored improperly, so they are no longer viable. You can test whether your seeds are in good condition using a technique popular among gardeners – simply roll a few seeds in a damp paper towel, seal it in a plastic bag, and then check the germination rate after a few days.

Another possible problem is that something went wrong with the conditions for growth. Did the dishes get too hot, too dry, or contaminated in some way?

**Analysis of Trends**

Looking at your graphs of average number of seeds germinated and average radicle lengths (Figures 2a and 2b), do you notice any trends? For example, does the toxicity of your test chemical appear to increase as the concentration increases, or does it stay the same from one concentration to the next? Are there any data that don’t seem to make sense? If so, make a note and try to think of any possible explanations for why they differ from your expectations.

**A Look at Variability**

The means for each treatment tell only part of the story. It is also useful to take a look at the individual data points to gauge how much variability exists within each treatment. In the case of lettuce seed bioassays, this means looking at the number of seeds sprouted per dish and the lengths of their radicles. Did the replicate dishes show similar numbers of seeds sprouting, and similar average radicle lengths? In bioassays, variability is inevitable because of the biological differences among organisms. Within the control group, for example, it is common to find some seeds that do not germinate, others that grow radicles only a few millimeters long, and others that reach 10 times this length.

Try graphing individual data points for each treatment. The wider the spread between data points, the greater the variability within that treatment. The more variability there is within each treatment, the less confident you can be that one treatment is different from another, even if the averages appear different on your bar graph (Figure 2a or b).

In addition to the inevitable variability caused by biological differences among organisms, your experimental techniques also will influence the variability of your data. At many steps in a bioassay, the measurements and decisions you make will affect your results. Were the serial dilutions carefully made according to directions? Were precise amounts of solution put into each Petri dish? Did one person measure radicle lengths, or did two or more people share this task? Did you stretch some radicles more than others while measuring them, or did you treat them all the same? Based on your experience with this bioassay protocol, what ideas do you have for reducing variability caused by measurement techniques?

**Estimating the TC50**

The next step in your data analysis is to figure out how to answer the question: How toxic is the solution or sample to the type of organism you tested? In bioassays there are two ways to report results: LC50, the lethal concentration that kills 50 percent of the test organisms, and TC50, the toxic concentration that causes organisms to grow 50% as well as the control...
group. In lettuce seed bioassays, the seeds don’t die – they either sprout and begin to grow, or they remain unspouted. So, in this case, use the TC50 to represent the concentration at which the lettuce seeds in the treatment grow approximately half as well as those in the control group.

For lettuce seed bioassays, there are two possible TC50s – one for germination rate and another for radicle length. Using Figures 2a and 2b, you can estimate which concentrations produce germination and growth rates roughly half those of the control group. If none of your concentrations produces rates that are close to half those of the control, it makes sense to report the TC50 as a range rather than a single number. For example, you might have to say that the TC50 is greater than or less than all the concentrations you tested, or that it lies somewhere between two of your tested concentrations.

**Drawing Conclusions about Toxicity**
After you have estimated the TC50 for your experiment, you will be able to use this number to make a statement about the toxicity of the substance you tested. Usually this statement will be something like:

*The TC50 for chemical X and lettuce seed radicle length is in the range of __ to __.*

If you have TC50 values for lettuce seeds exposed to other chemicals, you can use these numbers to rank which chemicals are most toxic to lettuce seeds. For example:

*The TC50 for chemical X is a smaller number than the TC50 for chemical Y. This means that chemical X can affect lettuce seed radicle growth at lower concentrations than chemical Y. Therefore, I conclude that chemical X is more toxic to lettuce growth than chemical Y.*

It is important to remember that lettuce seed bioassays will not help you to reach conclusions about toxicity to humans, because humans and lettuce are likely to respond very differently to chemical exposures. In order to use bioassays to predict toxicity to humans, you would need to use organisms such as laboratory rats that are known to provide a better model of human response to toxic chemicals.
# Lettuce Seed Dose/Response Bioassay Data Sheet

Name __________________________
Date __________________________

Chemical tested ___________________________
100% concentration ________________ mg/l
Length of experiment ________________ days

## Table 2a. Seed Germination Data

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Concentration (mg/l)</th>
<th># Seeds Germinated/Dish</th>
<th>Average # Seeds Germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## Table 2b. Radicle Length Data

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Radicle Length (mm)</th>
<th>Average Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01%</td>
<td></td>
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</tr>
<tr>
<td>0.1%</td>
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</tr>
<tr>
<td>1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Make bar graphs of the averages you calculated in Tables 1a and 1b:

**Figure 2b. Lettuce Seed Radicle Length**

**Figure 2b. Lettuce Seed Germination**
Some questions to consider:

1. Did at least 80% of the seeds in the control dishes germinate? If not, what would you recommend doing differently next time to try to get a better germination rate?

2. Did lettuce seed germination respond in a predictable way to concentration? Describe any trends you observed.

3. Do any of your data not fit the trends you observed? If so, can you think of any reasons why these data might lie outside the range you would expect?

4. What is your estimate of the TC50 based on your lettuce seed germination data?
   TC50 = ___
   What is your estimate of the TC50 based on your radicle length data?
   TC50 = ___
   Which shows a greater response to the chemical you tested: germination rate or radicle length? Describe any similarities or differences that you noticed in trends between these two indicators of toxicity.

5. What can you conclude about the toxicity of the substance you tested?
6. If other students carried out a dose/response experiment using the same chemical, did their data follow the same trends as yours?

7. Based on this experiment, would you say that lettuce seed germination or root length would provide a useful bioassay for water samples from the environment? Why or why not?

8. If you were going to repeat this experiment, what would you do differently? How might you improve the experimental design to reduce the variability of your data or lead to more reliable results?