EDVOTEK®

THE **BIOTECHNOLOGY**
EDUCATION COMPANY®

NEW

Edvo-Kit #
905

Edvo-Kit #905

The Dose Makes the Poison: Testing the Environmental Impact of Pollution

Experiment Objective:

Students explore pollution issues by developing and performing a bioassay that tests the effects of different toxicant concentrations on plant growth.

See page 3 for storage instructions.

1.800.EDVOTEK • fax 202.370.1501 • www.edvotek.com • info@edvotek.com

Table of Contents

Safety Data Sheets can be found on our website: **www.edvotek.com/safety-data-sheets**

Experiment Components

Component		Storage	Check (✓)
A	Radish Seeds	Room Temp.	❑
B	Plant Growth Medium	4° C Refrigerator	❑
C	Antifungal powder	-20° C Freezer	❑
D	Zinc Solution	Room Temp.	❑
E	Nickel Solution	Room Temp.	❑
F	Copper Solution	Room Temp.	❑
G	Salt Solution	Room Temp.	❑
H	Simulated Acid Rain Solution	Room Temp.	❑
I	Detergent (Tween)	Room Temp.	❑

This kit contains reagents for 10 groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

REAGENTS & SUPPLIES

Store all components below at room temperature.

Component	Check (✓)
• 50 ml Conical Tube	❑
• Cheese Cloth	❑
• Petri Dishes	❑
• 25 ml Serological Pipet	❑
• 15 ml Conical Tubes	❑
• 2 ml Microcentrifuge Tubes	❑
• 1.5 ml Microcentrifuge Tubes	❑
• Individually Wrapped 3.5 ml Transfer Pipets	❑
• Small Loops	❑
• ph Paper	❑

Requirements

- Microwave or hot plate
- Water Bath
- Pipet bulb or pipet pump (recommended)
- 70% Ethanol
- Sterile water
- Bleach
- Automatic micropipet, 200-1000 µl and tips (recommended)
- Parafilm, paper tape, or saran wrap
- String (recommended)
- Rulers
- Disposable lab gloves
- Safety goggles
- Growth Light (optional)
- Analytical Balance (optional)

Background Information

ENVIRONMENTAL TOXICOLOGY

In 1962 Rachel Carson published "Silent Spring" which described the detrimental effect of pesticides like DDT on the environment. The book helped to launch the multidisciplinary field of environmental toxicology. This field examines how chemicals move within the environment and how they affect biological systems. Common questions that environmental toxicologists ask are:

- Which chemicals are dangerous? How much exposure will cause harm?
- What are the negative effects of exposure to a particular chemical?
- What are the environmental and biological fates of different chemicals?

To answer these questions scientists must study biological systems from a variety of perspectives. At a small scale, they can study the cellular and molecular mechanisms of intoxication on individual organisms. At the ecosystem level, scientists can examine changes in diversity in an area that result from different species' susceptibilities to an introduced chemical. Figure1 illustrates the cascading effects of a toxin at different scales. Together, these studies provide clues about both the mechanism and broader impact of environmental toxins.

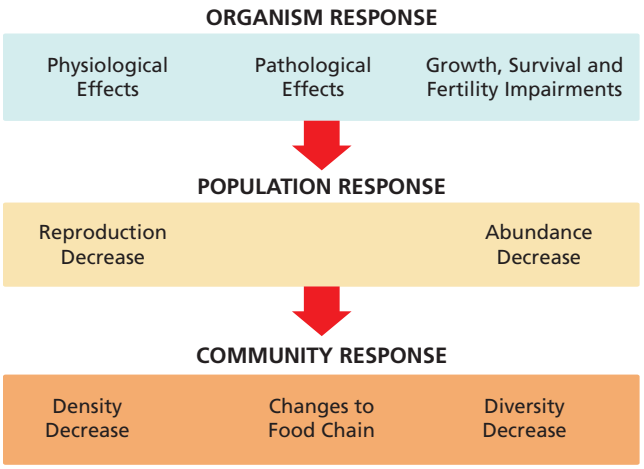


Figure 1: The effect of a toxin can be observed at different scales.

One area where environmental toxins can have a devastating effect is when they enter an ecosystem's or a municipalities' water supply. Water pollution can involve any change (chemical, biological, or physical) that has a harmful effect on the aquatic environment and any living organisms that depend on it. Scientists investigating water pollution often rely on a biological assay, or bioassay. This experiment measures the effects of a substance on living organisms, or indicator species, that serve as markers for the health of an ecosystem. Indicator species for bioassays need to be easily monitored in the lab or field and sensitive to changing environmental conditions. Popular choices are juvenile fishes, bivalve mollusks, and plant seeds. During a bioassay, several individuals of an indicator species are exposed to the substance being tested for a predetermined time and then compared to a control group that was not exposed to the substance. If the organisms in the test group show effects such as slow growth, reduced movement, or death, and if these effects are not seen in the control, then the substance being tested may be toxic.

When designing bioassays scientists must specify three types of variables: independent, dependent, and constant. The independent variable (Figure 2a) is the one that the scientists manipulate. Ideally there should be only one independent variable in order to know that this variable is causing the outcomes. The dependent variable or variables (Figure 2b) are the ones that the scientists observe. The dependent variables' values are caused by and dependent on the value of the independent variable, hence their name. Finally there are constant variables (Figure 2c). These are variables that might also affect the dependent variables and so are kept the same throughout the experiment.

A central concept of toxicology is that all chemicals are toxic at a certain amounts. This is summarized by the adage "The dose makes the poison." For example, trace amounts of many "toxic" heavy metals are necessary for the survival of certain organisms. Conversely, even pure water can be a poison when overconsumed. Therefore, toxicologists must consider the concentration, frequency and duration of exposure when researching the toxicity of a pollutant (Figure 3).

Pollutants in water are commonly measured and reported as parts per million (ppm). This is a notation that represents the proportion of a chemical in relation to the whole sample. It is similar to percentage except the scale is one million rather than one hundred. For example a solution that contains two grams of lead in one million grams of water is a 2 ppm solution. Because of certain properties of water 1 ppm is always equal to 1 mg of the pollutant in 1 L of water.

The other consideration when discussing toxins involves an organism's response to the dose. For example, mortality is an easy biological response to observe and track. Environmental toxicologists might measure a chemical's LD50, the amount of substance needed to kill 50% of the test population. However, scientists are also interested in sub-lethal responses such as a change in behavior or physiological impairment. Table 1 lists several common plant responses to chemical stress. Over time, these responses cause population changes including altered distribution, changes in age structure, and alterations

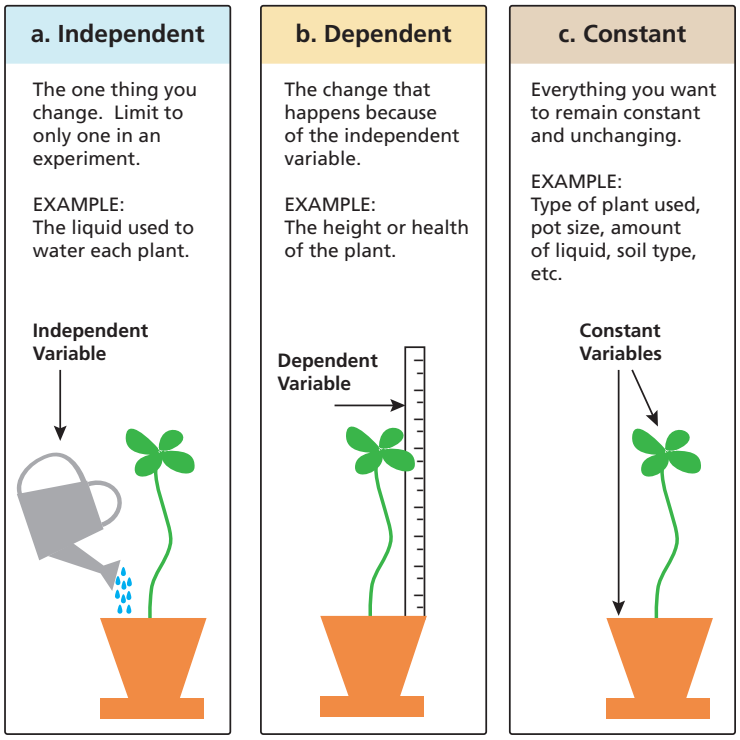


Figure 2: Types of Experimental Variables.

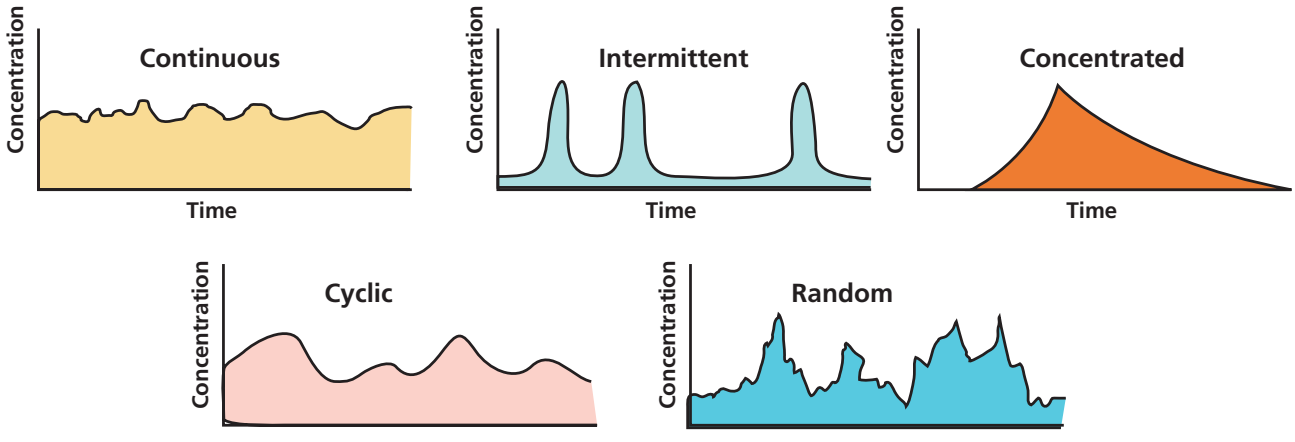


Figure 3: Pollutants enter an ecosystem in different ways.

to the gene pool. Together, these effects can lead to the eradication of a species from the environment and a decline in diversity.

Table 1: Plant injuries associated with toxin exposure.

Stage	Symptoms
Germination	• Low seed vigor/poor emergence • Swollen, twisted, or distorted seedlings • Leaf tip burn
Vegetative Plant Growth	• Distorted or discolored leaves • Death spots on leaves • Excessive slow growth • Small root systems
Reproductive Development	• Distorted and discolored fruits • Stunted pollen tube growth

Common Sources of Water Pollution

Water pollution is a major concern worldwide. The contaminants present in polluted water encompass a wide variety of chemical compounds, solid waste and microorganisms. Contamination enters the water supply through two main pathways (summarized in Figure 4). "Point source" water pollution refers to any contaminant that enters the water supply from a single, readily identifiable source, such as a manufacturing plant or water treatment facility. This waste, called effluent, is a complex material comprising sewage and/or chemical by-products. Since common practice is to release effluent into nearby bodies of water, the composition of this wastewater is strictly monitored and regulated by government agencies. In contrast, "nonpoint" pollution cannot be traced to a single, identifiable source — it often results from everyday activities. Typical nonpoint pollution arises from failing septic tanks, run-off from roads and farms, and atmospheric deposition from vehicle emissions.

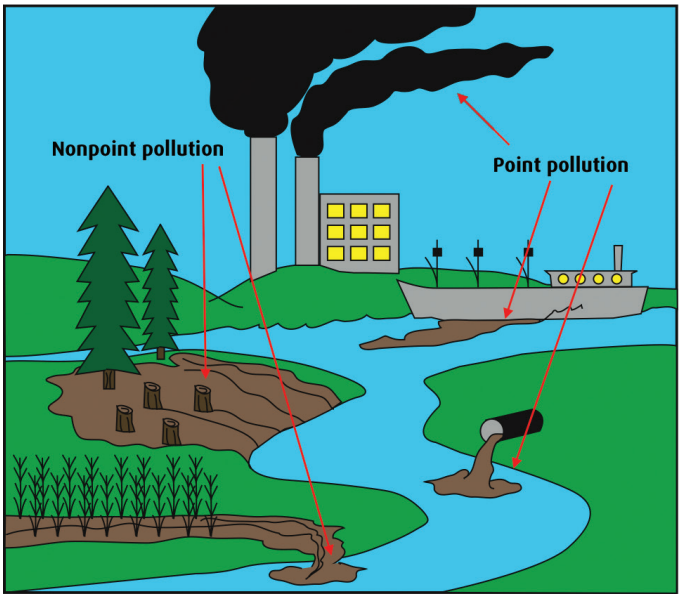


Figure 4: Point and Nonpoint sources of water contamination.

Both point and nonpoint pollution sources affect the local area, although they can also disturb the environment hundreds of miles away from the source (known as transboundary pollution). One common form of transboundary pollution is acid rain, which can harm plants, animals, and human infrastructure. Acid rain is caused by sulfur dioxide and nitrogen oxide emitted from burning fossil fuels. Most times, base nutrients in the soil like calcium, potassium, and magnesium can neutralize the acidity for a time. However, when ecosystems are exposed to chronic acidification these chemicals become depleted. Over time acid rain slows growth and injures plant life because the change in pH alters soil biology and chemistry - the hydrogen ions leach away essential nutrients while mobilizing other toxins like aluminum.

Metals are found naturally in the earth but become concentrated as a result of human activity. Exposure to cadmium, mercury, lead, and arsenic are of major public concern because of their known, serious, and adverse health effects. For example in 2016 the city of Flint Michigan declared a state of emergency after lead from aging pipes leached into the water supply. Table 2 lists additional common metal pollutants. To many people, heavy metal pollution is a problem associated with areas of intensive industry. However, roadways and automobiles are also a source of these pollutants. For example, brakes release copper, while tire wear releases zinc. On the road surface, most heavy metals become bound to the surfaces of road dust or other particulates. When it rains, the bound metals will either be dissolved or be swept off the roadway with the dust. In either case, the metals enter the soil or

are channeled into a storm drain. Plants are then exposed to the toxins through the uptake of water. Once within the plant, the toxins can bind and inhibit cellular components such as structural proteins, enzymes, and nucleic acids.

Salinization is the gradual build up of salt in soil and water. Salt can accumulate from many sources, including run-off of compounds used to remove snow and ice from the roads or improper irrigation techniques. In sufficient concentrations salts pose a risk to plants, animals and the aquatic environment. As the salt concentration in soil rises water does not flow as freely into cell roots, causing drought-like conditions for the plant. In addition, when salt dissolves in water, sodium and chloride ions separate and can be transported to the leaves where they accumulate and cause leaf scorch - a browning of tissue and yellowing of veins.

Table 2: Classification of metals according to their toxicity.

Non Toxic		Low Toxicity		Moderate to High Toxicity			
Aluminum	Magnesium	Barium	Praseodymium*	Actinium*	Indium	Polonium	Uranium
Bismuth	Manganese	Cerium*	Promethium*	Antimony	Iridium*	Radium*	Vandium
Calcium	Molybedenum	Dysprosium*	Rhenium*	Beryllium	Lead	Ruthenium*	Zinc
Iron	Potassium	Erbium*	Rhodium*	Boron	Mercury	Silver	Zirconium*
Lithium	Rubidium	Europium*	Samarium*	Cadmium	Nickel	Tantalum*	
	Sodium	Gadolinium*	Scandium	Chromium	Niobium*	Thallium	
		Gallium*	Terbium*	Cobalt	Osmium*	Thorium	
		Geramanium	Thulium	Copper	Palladium	Titanium*	
		Gold*	Tin*	Hafnium*	Platinum	Tungsten*	
		Holmium*	Ytterbium*				
		Neodymium*	Yttrium				

* Denotes metals that normally do not dissolve in water.

This lab focuses on the effect of common water pollutants on the health of sprouting *Raphanus sativus* (radish) plants. Students will explore independent, dependent, and constant variables in a self-directed bioassay. Each group will design and execute a bioassay using several concentrations of a pollutant to determine its environmental hazard. Common toxins including zinc, nickel, copper, salt, and acidified water are provided and can be tested with this bioassay. Alternatively, students can also test the effects of regionally significant point and nonpoint pollution for their effects on plant life. For instance, students can research local refineries or factories to form a hypothesis about what type of waste may be released into local rivers (point pollution). Non-point pollution from improper disposal of common cleaning or personal care products can also be explored. After performing the experiment, students analyze the data to determine the concentration of the chemical that is toxic to plant life.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students explore pollution issues by developing and performing a bioassay that tests the effects of different toxicant concentrations on plant growth.

LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. Always wash hands thoroughly with soap and water after handling contaminated materials.

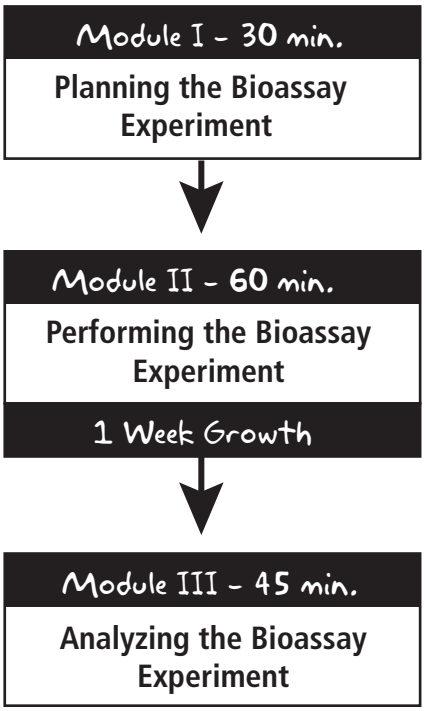
LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:
- Record your observations.

Following the Experiment:
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.



Module I: Planning the Bioassay Experiment

In this lab you will design and conduct a bioassay to determine at what concentration an environmental pollutant is toxic to plant growth. Use the following worksheet to develop your experiment before beginning Module II.

Ask a question: This question is answered using experimentation and factual reasoning. In the question you should specify what pollutant you are interested in testing and what indicator species you plan on using. Research potential toxicants or use one of the five provided with the kit. For this experiment we have provided radish seeds but you may choose an alternative plant species if desired.

Example: "At what concentration is lead toxic to radish plants?"

_____

_____

_____

_____

Form a hypothesis: This is one possible answer to your question and a prediction of what you think will happen. Remember that you are running a bioassay where you observe the response of an organism to a changing chemical dose.

Example: "Radish seed germination and radish plant growth will decrease with increasing concentrations of lead."

_____

_____

_____

_____

Choose an independent variable: This is the factor you will change in your experiment. Ideally, there is only one independent variable. In this bioassay the chemical dose is the independent variable.

Example: "The concentration of lead."

_____

_____

Module I: Planning the Bioassay Experiment, continued

Choose dependent variable(s): These are the factors that you predict will change as a result of variation in your independent variable. There can be several. Use Figure 5 to anticipate what changes can be observed and quantified. Be as specific as possible.

Example: "We predict that the number of seeds that germinate and the length of their roots will change as a result of varying lead concentrations. We define germination as when the radicle (primary root) appears and will measure the root length to the nearest mm."

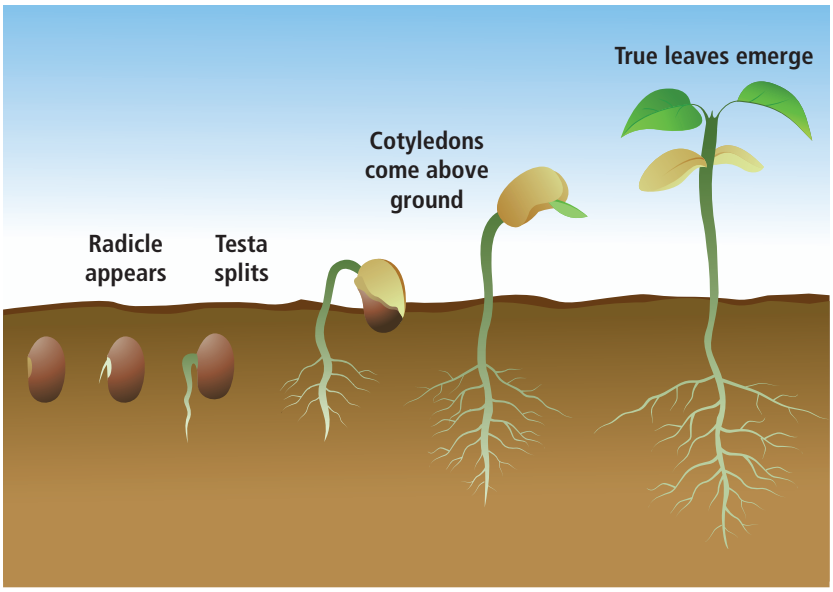


Figure 5: Key steps in plant germination and growth.

Specify the constants: These are the factors that do not change. Petri dish orientation (vertical vs. horizontal) and growing time should be decided here. We strongly suggest that plants are grown on 15 ml of supplemented agar gel comprised of 14 ml plant growth media and 1 ml of liquid pollutant.

Example: "Plants will be grown for one week in vertical petri plates. Each plate will have the same temperature, humidity, and light exposure. 14 mL of plant growth media and 1 ml liquid pollutant will be added to each plate."

Module I: Planning the Bioassay Experiment, continued

Plan the treatment: The independent variable should be tested on at least two groups, with each group receiving different concentrations of pollutant. We recommend testing at least three concentrations. It is often convenient to construct a dilution series for the treatment groups. We found a 1:10 dilution series created a good level of contrast between conditions. See note below if your group decides not to use a 1:10 dilution series. The provided heavy metal pollutants will create a plate with either a 1,000 ppm (copper and nickel) or 10,0000 ppm (zinc and salt) concentration when 1 mL of the concentrated solution is combined with 14 mL of the plant growth media. The acid rain solution is provided at a starting pH of 1.35. For the simulated acid rain pollutant you will rank the acidity of each experimental condition (e.g. low, medium, and high) in Module I and then measure the pH of your dilutions in Module II.

Example: "Experimental Conditions 1 – 100 ppm Lead. Experimental Condition 2 – 10 ppm Lead. Experimental Condition 3 - 1 ppm Lead."

Experimental Condition 1	
Experimental Condition 2	
Experimental Condition 3	

NOTE: Depending on your group's chosen experimental concentrations you may need to perform additional calculations to determine how to prepare each solution. Do this before beginning Module II. General instructions are given in Appendix B.

Choose a control: This is the group that will serve as a standard of comparison. If the control group is treated very similarly to the experimental groups, it increases our confidence that any difference in outcome is caused by the presence of the experimental treatments.

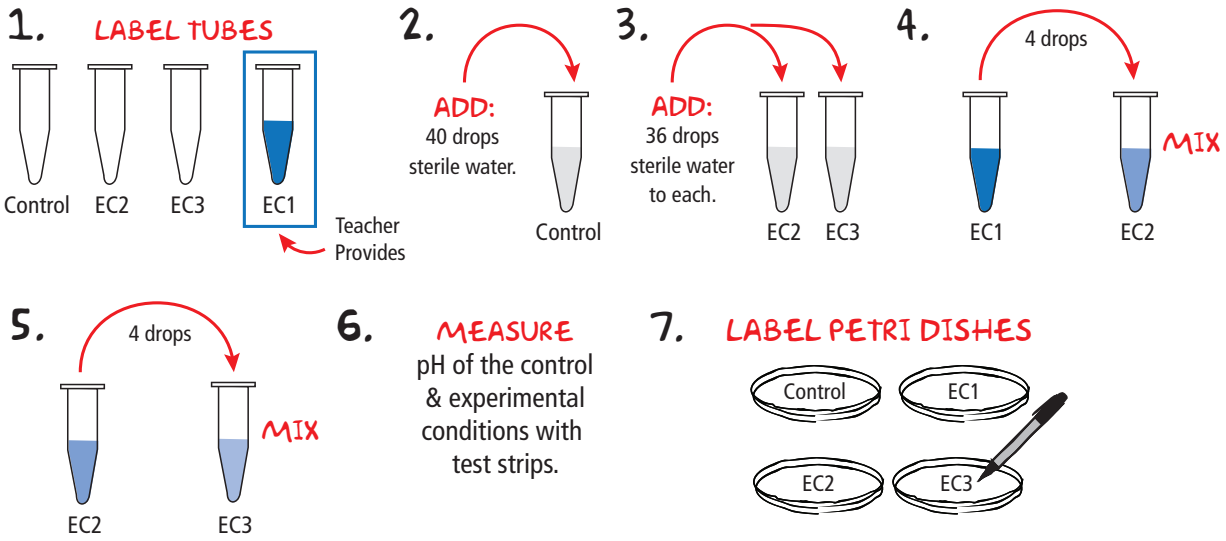
Example: "Control – 0 ppm Lead (pure water)".

Control	

Decide on the number of replicates: These are individuals that are exposed to exactly the same conditions in an experiment. In this experiment the number of replicates will be the number of seeds planted in a petri dish. Additional replicates often increase the accuracy and reliability of summary statistics. However, each replicate requires resources such as space and nutrients.

Example: "5 seeds per plate."

Module II: Performing the Bioassay Experiment



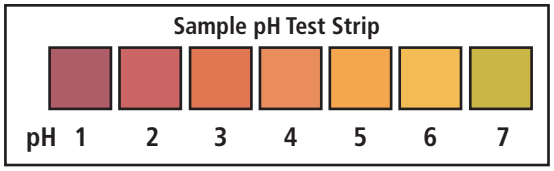
PREPARING GROWTH PLATES

In steps 1 through 6, your group will create solutions for each of the three experimental conditions and the control. Instructions and volumes for these steps are given for a bioassay involving three experimental conditions and a 1:10 dilution series. If you are using a different experimental set up, refer to the calculations made in Module I and Appendix B.

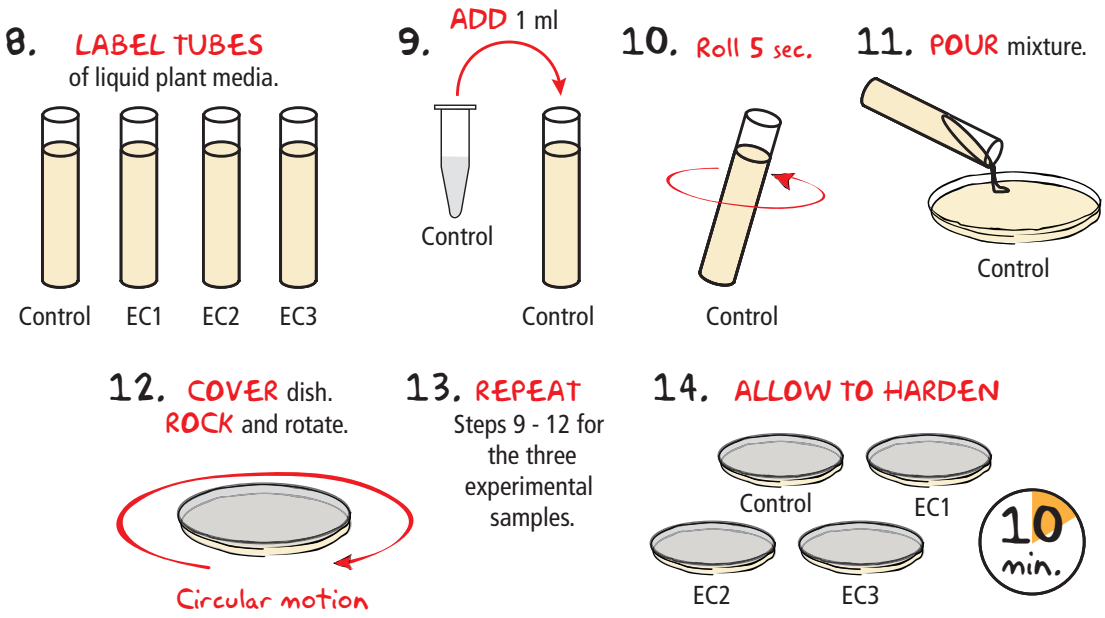
1 **LABEL** three empty 2 ml tubes as control, EC2, and EC3. Your teacher will provide an EC1 tube which is the pollutant at the highest concentration.
2. **ADD** 40 drops (1.6 ml) of sterile water to the control tube using the transfer pipet.
3. **ADD** sterile water to tubes EC2 and EC3. If you are doing a 1:10 dilution series the volume will be 36 drops (1.44 ml). This volume, as well as the volumes in steps 4 and 5, will be different if you are using a different dilution factor or other concentrations.
4. Using the transfer pipet **ADD** 4 drops (160 µl) of experimental condition 1 to the experimental condition 2 tube. **MIX** by pipetting up and down.
5. Using the transfer pipet **ADD** 4 drops (160 µl) of experimental condition 2 to the experimental condition 3 tube. **MIX** by pipetting up and down.
6. If your group is testing acid rain as a pollutant, **MEASURE** the pH of the control and each experimental condition using the sample test strip, at right.
7. **LABEL** the bottom of 4 petri dishes with (a) your group I.D., (b) the pollutant, (c) the experimental condition number or "Control", and (d) the date.

Table 3: 1:10 Dilution Series

Experimental Condition 1 (EC1) 1:1 dilution	Experimental Condition 2 (EC2) 1:10 dilution	Experimental Condition 3 (EC3) 1:100 dilution
1.6 ml of concentrate pollutant	1.44 ml of sterile water + 160 µl of 1:1 dilution	1.44 ml of sterile water + 160 µl of 1:10 dilution



Module II: Performing the Bioassay Experiment, continued



8. **OBTAIN** four 15 ml tubes with liquid plant media from the 60° C waterbath. Work quickly as the media will cool and begin to solidify. **LABEL** each tube EC1, EC2, EC3, and control.
9. **ADD** 1 ml of control solution to the plant media tube labeled "control".
10. **ROLL** the tube between your palms for 5 seconds to mix the solution with the media. Avoid introducing bubbles into the media.
11. Quickly **POUR** the mixture onto the dish labeled "control".
12. **COVER** the dish and lift it from the bench. Holding the plate in your hands, gently **ROCK** and rotate the dish in a circular direction to disperse the plant media evenly across the dish.
13. **REPEAT** steps 9 through 12 for the three experimental samples.
14. **ALLOW** the plant media to harden for at least 10 minutes.

NOTE:
Work quickly through steps 8 through 13 to prevent media from prematurely solidifying.

OPTIONAL STOPPING POINT:
Petri plates can be stored a 4° C for up to one week. Keep plates closed, horizontal, and wrapped in plastic (saran wrap or the original plastic sleeve) to avoid drying and contamination.

Tips for keeping a sterile environment:

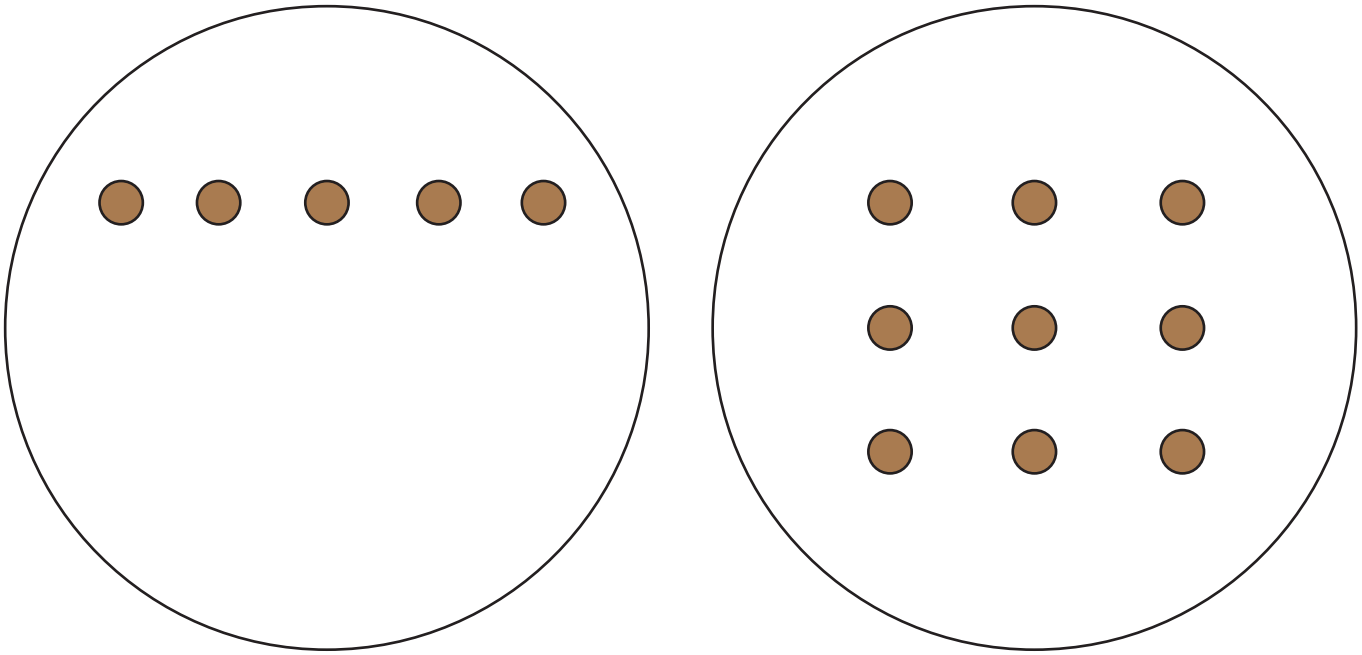
- It is important to prevent contamination of the plant growth media by limiting contact with air. Once the media has been added keep the lid on petri dishes whenever possible.
- Sterilize counter top with 70% EtOH.
- Wear gloves.

Module II: Performing the Bioassay Experiment, continued

PLANTING AND GROWING THE SEEDS

15. Using the loop gently **PLACE** seeds in each petri dish. The seeds should rest on the surface of the media for at least 5 minutes. A template is provided below for five and nine seed placement.
16. Carefully **SEAL** each dish with parafilm, saran wrap, or tape around edge to help keep the environment moist. Even with gentle handling, seeds may move out of place. This is okay.
17. **PLACE** the dishes in an evenly lit location where they can grow undisturbed throughout the experiment. Radish seeds prefer to grow in direct sunlight, in a growth chamber or under a grow light.
18. **ALLOW** seeds to germinate and grow for an agreed number of days. If light exposure is uneven, periodically change the position of the dishes to ensure each experimental condition receives similar levels of light.

NOTE:
The media on your plates is fragile, DO NOT attempt to embed the seeds into the media.



Module III: Analyzing the Bioassay Experiment

COLLECTING THE BIOASSAY DATA

1. Retrieve the four dishes and **OBSERVE** their overall appearance. **RECORD** your observations, consider drawing or taking a picture of each dish.

2. Unseal each dish and remove the lid to examine each seedling. **MEASURE** and **RECORD** your dependent variables. Use the table, below, as a model for recording the data. If seedlings have curved during initial growth, stretch out the plant or use a string to measure the full length.

Dependent Variable Measurements After ___ Days

	Seed 1	Seed 2	Seed 3	Seed 4	Seed 5	Seed 6	Seed 7	Seed 8	Seed 9	Average
Control _____										
Experimental Condition 3 _____										
Experimental Condition 2 _____										
Experimental Condition 1 _____										

Table 4

INTERPRETING THE BIOASSAY DATA

1. **CHECK** your control to confirm that (a) seeds were grown under suitable conditions and that (b) comparison between group's results is appropriate.

   Working with living organisms inevitably introduces variability. However, if fewer than 80% of the seeds in your control dish germinated something may have gone wrong in your experiment. Also compare the dependent variable averages for your control with another groups. Because all control plates should have water in the place of a pollutant these values should be similar.

2. **CREATE** bar graphs of your results using the average values from the dependent variables. Data can be plotted by hand or by using a computer graphing program.

   Use these graphs to identify any trends or unexpected results. Does the toxicity of your pollutant increase/decrease/remain the same from one concentration to the next? Are there any changes that you did not expect? What could be a possible explanation for these changes?

3. **EXAMINE** the variability.

   Average values tell an important story but it is also useful to look at the individual data points to see how much variability existed within an experimental condition. As mentioned above bioassays will inevitably have some variability because of biological differences between individuals.

Module III: Analyzing the Bioassay Experiment, continued

Human error – such as one person measuring a length slightly differently than another – can also introduce variability. Large differences between experimental conditions are less meaningful when there is also a high level of variability within experimental conditions.

(Optional) CALCULATE the standard deviation for each experimental condition and use these values to **ADD** error bars to the graphs. Error bars that do not overlap indicate that the difference between two conditions is likely not due to chance. Conversely, error bars that do overlap indicate that any difference between the two conditions should be more cautiously interpreted.

The standard deviation of a sample is known as S and is calculated using the formula:

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

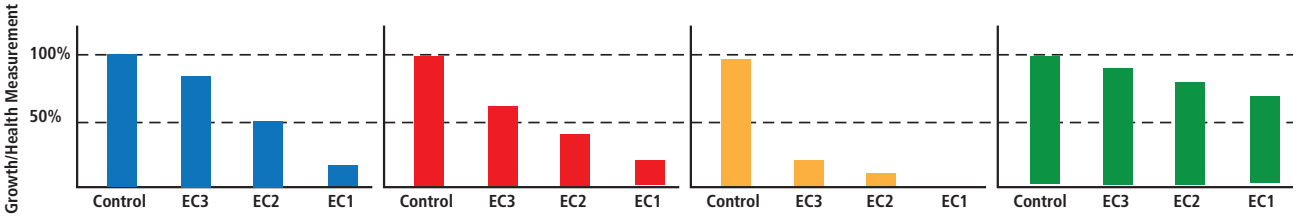
Where x represents each value in the population, x is the mean value of the sample, Σ is the summation (or total), and n-1 is the number of values in the sample minus 1.

The standard deviation can be calculated using an appropriate software program or online resource. It can also be calculated by hand in four steps:

(a) Record the average of all the replicates.
(b) Take the first replicate value and subtract the average from step a. Then square the result. Repeat this for all replicates and record the result.
(c) Add the results from step b and then divide by the number of replicates minus one.
(d) Take the square root of the number from step c.

4. **(Optional) ESTIMATE** a TC50 value.

The TC50 value is the concentration that causes a 50% drop in the growth or health of the test organisms. Use your graphs to estimate the concentration that produces germination or growth rates roughly half of the control groups. In many cases, this value will be near one of your test concentrations (blue graph) which will allow you to estimate the TC50 or between two different test concentrations (red graph) which will allow you to give a potential range for the TC50. However, in some cases, the concentrations tested will be too strong (yellow graph) or too weak (green graph). In these cases, you can still conclude that the TC50 is lower (yellow)/ higher (green) than the tested concentrations and that additional tests are needed.



5. **COMPARE** results with other student groups and if possible rank each pollutant from the most to least toxic for radish seeds.

Study Questions

1. In the field of environmental toxicology what is a bioassay experiment?
2. Name and define the three main categories of experimental variables.
3. What does the saying "the dose makes the poison" mean?
4. What does ppm stand for? Calculate the ppm concentration of lead in a solution of water if 1 milligram is dissolved in 2 L of water.
5. What is the difference between point source and nonpoint source pollution? Why might the EPA have different strategies to control these two types of water pollution?
6. Depending on your pollutant answer one of the following questions:

   (a) What causes acid rain? Why are the effects of acid rain not always seen immediately in an ecosystem?

   (b) How do metal pollutants such as copper, zinc, and nickel effect plant cells? Name one way that they can be introduced into the environment.

   (c) Name two sources of salt pollution. How does salt negatively affect plant life?

   (d) What pollutant did you bring from home? How did you expect the pollutant to impact plant growth?

Instructor's Guide

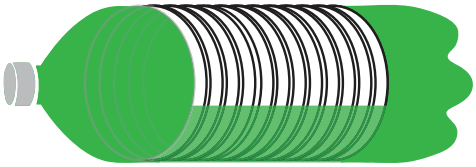
OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
Module I: Planning the Bioassay Experiment	Read through recommendations table on the next page.	Any time before the class period.	15 min.
	Remind students to bring in locally relevant pollutants they'd like to investigate.	Any time before the class period.	---------
Module II: Performing the Bioassay Experiment	Sterilize water.	Any time before the class period.	30 - 45 min.
	Prepare and aliquot reagents.	Any time before the class period.	15 min.
	Sterilize seeds.	The day of the class period.	30 min.
	Prepare the plant growth media.	Immediately before the class period.	30 min.
Module III: Analyzing the Bioassay Experiment	Gather graphing paper or obtain access to computers.	Any time before the class period.	---------

Module I Pre-Lab Preparations

TIPS AND TRICKS

Choosing an independent variable	In this kit we provide material to manipulate the concentration of zinc, nickel, copper, and salt. We also provide an "acid rain" solution that can be used to change the pH value of the media. Each of these five pollutants can be tested by two groups. In addition we have tested caffeine, dish soap, and instant hand sanitizer. House cleaners, personal care products, over the counter medicines, samples from local stream waters, and other safe chemicals are all possible pollutants to test. To determine the concentration of any additional pollutants see Appendix B. Please note that the provided plant media contains polymerized agar, which can collapse at low pH levels or in the presence of concentrated detergents. If using either of these as pollutants we recommend keeping pH levels above 2.5 and detergent levels less than 1000 ppm (0.1%).
Choosing dependent variables	Root/Radical length and germination success are the most tested variables for plant bioassays. Other potential measurements are shoot length, total plant length, root to shoot ratio, and final plant mass. Decide on whether these variables will vary from group to group or be universal in order to enable between group comparisons. If you decide on class wide variables be sure to specify how the class defines germination. In addition, root structure in radish seedlings is usually tap root but can sometimes be fibrous. Before performing the experiment, decide whether to combine the measurements of multiple long roots.
Defining the constants	Plate orientation: We suggest a horizontal orientation if using a soaps/detergent pollutant as the agar can collapse when vertical. However, vertical dishes allow for easy viewing and measurements of the growing plants as well as more compact storage. If you are going to keep the dishes vertical you will need to create a chamber to hold the petri dishes. We found the empty boxes of 1000 µL pipet tips useful. A 1 liter soda bottle that has been cut as bellow will also work.  Volumes: For best results, we recommend preparing petri plates containing 1 ml of pollutant and 14 ml of plant growth media. Using these volumes, this kit provides enough plant growth media for forty growth plates. Light: Variation in light exposure can significantly effect the rate of growth in each plate. Consider regularly changing plate positions if the light source is sun from a window. Alternatively, creating a plant growth chamber (Appendix A) or using a growth light can help keep light intensity constant across plates. Temperature: Radish seeds germinate and grow best in temperatures between 12°C and 24°C. Be sure your classroom maintains these temperatures over nights and weekends. Growth time: Radish seeds usually germinate within three days. They then grow rapidly and uninhibited can reach lengths of 15 cm in one week. Plants will grow even faster in a growth chamber. The risk of contamination increases with longer growth times.
Determining the number of replicates	We found five seeds to be optimal for vertical plates and nine seeds to be optimal for horizontal plates. We have included templates for these in Module II instructions. This kit provides enough seeds for forty plates with 9 seeds each.

Module II Pre-Lab Preparations

Sterilize water

500 ml of sterile water will be required for this experiment. If you have access to an autoclave we recommend sterilizing the water at 121° C for 40 minutes. Alternatively, water can be mostly sterilized by boiling for 15 minutes. If you are using a microwave to boil water, reduce the risk of superheating by only boiling for 5 minutes at a time, adding a clean and microwave safe object to the water before beginning, and carefully tapping the side of the container before taking it out of the microwave. A final option is purchasing commercial distilled water.

Prepare and Aliquot Reagents

1. **ALIQUOT** 5 ml of sterile water to ten 15 ml tubes and **LABEL** each tube "sterile water."
2. Prepare stock pollutant concentrate for all ten groups.
   a. **ALIQUOT** 1.8 ml of Zinc Solution (Component D) into two 2 ml snap top tubes. **LABEL** each tube "10,000 ppm Zinc".
   b. **ALIQUOT** 1.8 ml of Nickel Solution (Component E) into two 2 ml snap top tubes. **LABEL** each tube "1,000 ppm Nickel".
   c. **ALIQUOT** 1.8 ml of Copper Solution (Component F) into two 2 ml snap top tubes. **LABEL** each tube "1,000 ppm Copper".
   d. **ALIQUOT** 1.8 ml of Salt Solution (Component G) into two 2 ml snap top tubes. **LABEL** each tube "10,000 ppm Salt".
   e. **ALIQUOT** 1.8 ml of Simulated Acid Rain Solution (Component H) into two 2 ml snap top tubes. **LABEL** each tube "Acid 1.35 pH".
   f. **ALIQUOT** any additional pollutant and **LABEL** appropriately.

Sterilize Seeds

Sterilize seeds the day of Module II as these seeds quickly germinate.

1. Create the sterilization solution by **MIXING** 15 ml bleach, 15 ml sterile water, and the entire tube of Tween (Component I) in the 50 ml conical.
2. **PREPARE** six cheesecloth squares. Each square should be at least 5x5 cm and 3 to 4 sheets thick.
3. **ADD** seeds (Component A) to the 50 mL conical tube.
4. **INCUBATE** for 5 to 10 minutes. Gently rock the tube from time to time to rotate the seeds.
5. Place a cheesecloth square over the top of the tube and **POUR** out the sterilization solution.
6. **REFILL** tube with sterile water.
7. **WASH** the seeds in sterile water by gently rocking the tube for 1 minute.
8. **PLACE** a new cheesecloth square over the top of the tube and **POUR** out the water.
9. **REPEAT** step 6 through 8 at least four more times.
10. **TRANSFER** the appropriate number of seeds to ten 1.5 mL centrifuge tubes using a sterile loop. (25 for groups using vertical plates, 45 for groups using horizontal plates.)

Module II Pre-Lab Preparations, continued

Prepare the Plant Growth Medium

1. **SET** a waterbath to 60° C.
2. **LOOSEN**, but do not remove, the caps on the bottles of plant growth media (Component B) to allow for steam to vent during heating. ***CAUTION: Failure to vent the cap before heating may cause the bottle to break or explode.***
3. **MICROWAVE** all bottles of plant growth media on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottles from the microwave and **MIX** by swirling the bottles. Continue to **HEAT** the media in 30-second intervals until the agar is completely dissolved (the yellow-colored solution should be clear and free of small particles).
4. Allow the media to **COOL** to 60° C.
5. **ADD** one tube of antifungal powder (Component C) to each bottle and mix by swirling.
6. **DISPENSE** 14 ml of media per 15 ml screw-top conical tube (4 tubes per group, 40 tubes total).
7. **PLACE** tubes of melted media in the 60° C waterbath to keep the agar from solidifying.

NOTE:
The agar MUST be kept warm at all times. If the media does solidify in the bottle or in the 15 ml screw-top tubes it can be carefully reheated in the microwave using 10-second intervals. Remember to loosen caps before heating.

FOR MODULE II
Each Student should receive:
- Four 100 mm petri dishes
- Three 2 ml microcentrifuge tubes
- Individually wrapped transfer pipet
- Small loop
- One tube of Radish seeds
- 5 ml sterile water
- 1.8 ml pollutant
- Parafilm, paper tape, or saran wrap
- Four 15 ml tubes with warm plant growth medium
- For acid rain test groups only: 4 pH strips

Tips for Keeping a Sterile Environment

- It is important to prevent contamination of the plant growth media by limiting contact with air. Once the media has been added, keep the lids on the petri dishes whenever possible.
- Sterilize the counter top with 70% EtOH.
- Wrap each small loop in saran wrap or similar protective covering once it has been removed from the bag.
- Have students wear gloves.

Module III Pre-Lab Preparations

In this section, students calculate averages, standard deviations, and approximate TC50 values using graphing paper and a standard calculator or a graphing program. The data can also be analyzed using ANOVAs but will require additional guidance and computer resources.

Experiment Results and Analysis

MODULE I: PLANNING A BIOASSAY EXPERIMENT

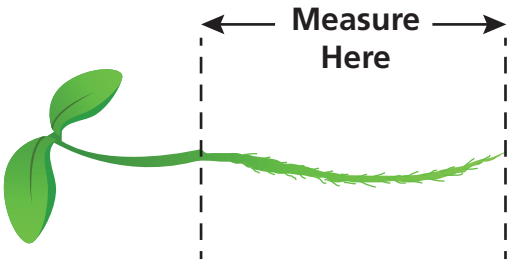
These results are representative. Responses will be unique to each student group.

Ask a Question: "At what concentration does copper become toxic to radish plants?"

Form a Hypothesis: "Radish seed germination and radish plant growth will decrease with increasing concentrations of copper."

Choose an Independent Variable: "The concentration of copper."

Choose Dependent Variable(s): "The number of seeds that germinate and the length of their roots. We define germination as when the radicle (primary root) appears and will measure the root length to the nearest mm as shown."



Specify the Constants: "Temperature, humidity, light intensity, vertical dish orientation, 1 week growing time, 14 ml of plant growth media and 1 ml liquid pollutant."

Plan the Conditions:

Experimental Conditions 1	"1,000 ppm of copper"
Experimental Conditions 2	"100 ppm of copper"
Experimental Conditions 3	"10 ppm of copper"

Choose a Control:

Control	"0 ppm of Copper"

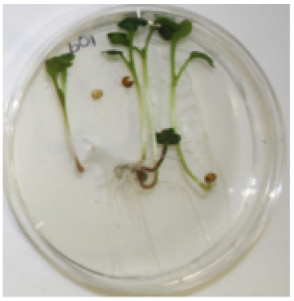
Decide on the Number of Replicates: "5 seeds per plate."

Experiment Results and Analysis

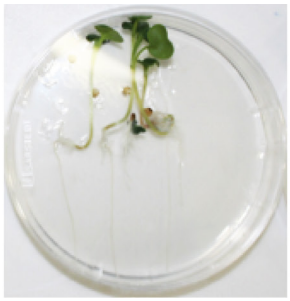
MODULE III

These are representative results from an in house bioassay where radish seeds were grown at different copper concentrations for seven days. The effects of copper are shown in detail. Root length is used to illustrate an effective graphing and statistical analysis approach.

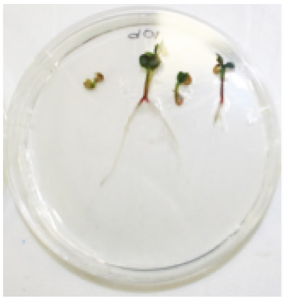
Control



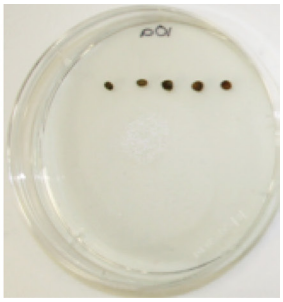
Copper 10 ppm



Copper 100 ppm



Copper 1,000 ppm



Dependent Variable: Root Length (mm) After 7 Days

	Seed 1	Seed 2	Seed 3	Seed 4	Seed 5	Average	Std. Dev.
Copper_Control	80	49	85	79	68	72.2	14.4
Copper_10 ppm	20	60	67	61	65	54.6	19.6
Copper_100 ppm	1	8	9	31	29	15.6	13.5
Copper_1,000 ppm	0	0	0	0	0	0.0	0.0

Dependent Variable: Stem Length (mm) After 7 Days

	Seed	Seed 2	Seed 3	Seed 4	Seed 5	Average	Std. Dev.
Copper_Control	64	1	41	49	64	43.8	25.9
Copper_10 ppm	7	45	38	50	30	34.0	16.9
Copper_100 ppm	4	13	12	13	18	12.0	5.1
Copper_1,000 ppm	0	0	0	0	0	0.0	0.0

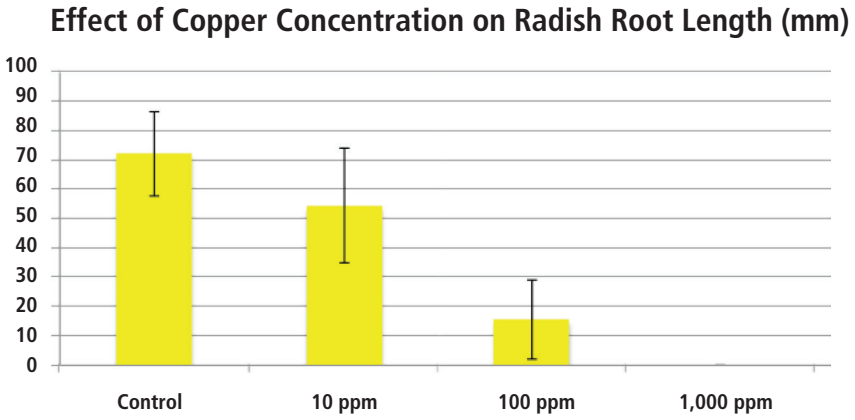
Dependent Variable: Plant Wet Weight (mg) After 7 Days

	Seed 1	Seed 2	Seed 3	Seed 4	Seed 5	Average	Std. Dev.
Copper_Control	160.5	88.3	176.6	144.5	128.4	139.7	33.9
Copper_10 ppm	30.1	93.4	101.7	96.5	98.6	84.1	27.1
Copper_100 ppm	2.1	16.8	18.9	64.9	60.8	32.7	25.3
Copper_1,000 ppm	11.8	14.4	10.8	9.6	13.2	12.0	1.7

Dependent Variable: Germination After 7 Days

	Seed 1	Seed 2	Seed 3	Seed 4	Seed 5	%	Std. Dev.
Copper_Control	yes	yes	yes	yes	yes	100	NA
Copper_10 ppm	yes	yes	yes	yes	yes	100	NA
Copper_100 ppm	yes	yes	yes	yes	yes	100	NA
Copper_1,000 ppm	no	no	no	no	no	0	NA

Experiment Results and Analysis



In this experiment, the average root length in the control group was 72.2 mm. Consequently, the TC50 is the concentration that results in an average root length of 36.1 mm. This concentration falls between 10 ppm (average root length of 54.6 mm) and 100 ppm (average root length of 15.6 mm).

*Calculating Standard Deviation for Control:
(1) Average of replicates: (80+49+85+79+68)/5 = 72.2
(2) Replicate 1: $(80-72.2)^2$ = 60.84
Replicate 2: $(49-72.2)^2$ = 538.24
Replicate 3: $(85-72.2)^2$ = 163.84
Replicate 4: $(79-72.2)^2$ = 46.24
Replicate 5: $(68-72.2)^2$ = 17.64
(3) Combine replicates divided by four: (60.84+538.24+163.84+46.24+17.64)/4 = 206.7
(4) Square root of step 3 results: 14.38

*Calculating Standard Deviation for Copper 10 ppm:
(1) Average of replicates: (20+60+67+61+65)/5 = 54.6
(2) Replicate 1: $(20-54.6)^2$ = 1197.16
Replicate 2: $(60-54.6)^2$ = 29.16
Replicate 3: $(67-54.6)^2$ = 153.76
Replicate 4: $(61-54.6)^2$ = 40.96
Replicate 5: $(65-54.6)^2$ = 108.16
(3) Combine replicates divided by four: (1197.16+29.16+153.76+40.96+108.16)/4 = 305.84
(4) Square root of step 3 results: 19.55

*Calculating Standard Deviation for Copper 100 ppm:
(1) Average of replicates: (1+8+9+31+29)/5 = 15.6
(2) Replicate 1: $(1-15.6)^2$ = 213.16
Replicate 2: $(8-15.6)^2$ = 57.76
Replicate 3: $(9-15.6)^2$ = 43.56
Replicate 4: $(31-15.6)^2$ = 237.16
Replicate 5: $(29-15.6)^2$ = 179.56
(3) Combine replicates divided by four: (213.16+57.76+43.56+237.16+179.56)/4 = 182.8
(4) Square root of step 3 results: 13.52

Please refer to the kit insert for the Answers to Study Questions

Please refer to the kit insert for the Answers to Study Questions

Appendices

A Creating a Plant Growth Chamber
B Calculating Concentrations
C Representative Experimental Results

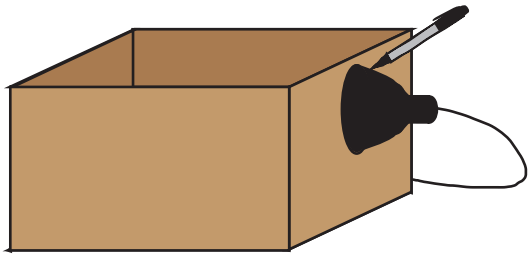
Safety Data Sheets:
Now available for your convenient download on **www.edvotek.com/safety-data-sheets**

Appendix A
Creating a Plant Growth Chamber

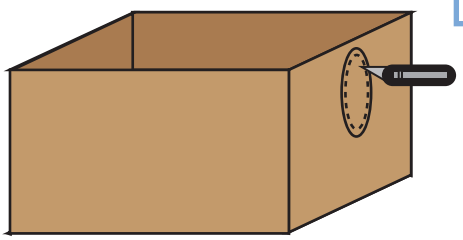
Materials Needed:
- Open top cardboard box (18x18x18 or similar)
- Marker
- Aluminum foil
- Tape
- Reflector lamp
- 23W Compact Fluorescent bulb (CFL bulb)
- Razor or sharp knife

A plant growth chamber can help ensure success with Module II of this experiment.

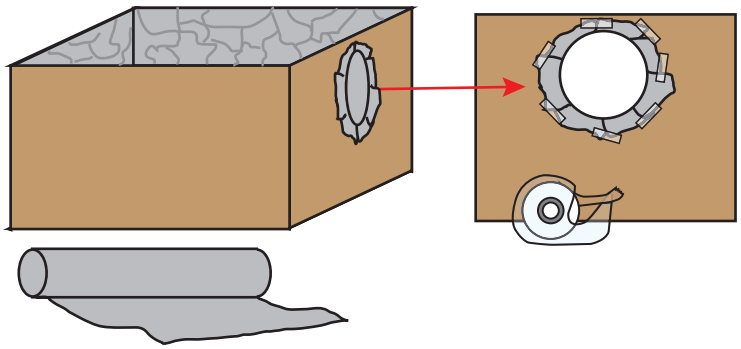
1. Place the reflector lamp on the top center of one of the sides of a small cardboard box. Use a marker or pen to trace a circle around the lamp.



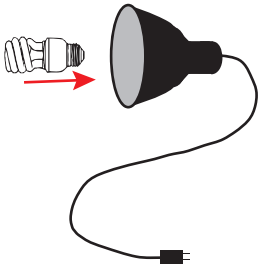
2. Cut a circle 1 cm smaller than the traced circle.



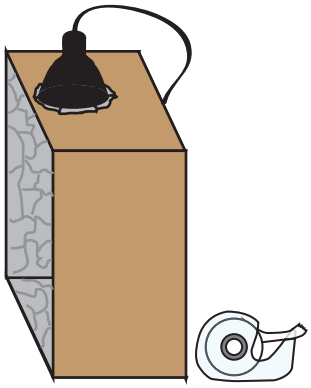
3. Use aluminum foil to line the inside of the box. Use extra pieces to cover the hole. Tape foil in place.



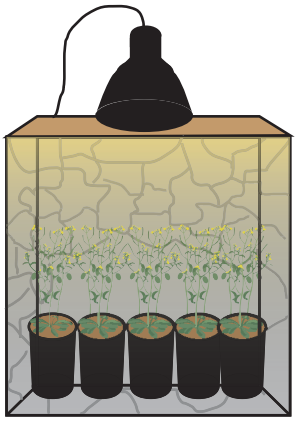
4. Screw the CFL bulb into the lamp.



5. Place the lamp over the hole. Secure with tape.



6. The plant growth chamber is ready to go! Use a small box or riser to adjust the proximity of the potted seeds to the light depending on the seed type and the lighting requirements.



Appendix B
Calculating Concentrations

Students may want to choose specific concentrations or test additional pollutants that are locally relevant. In these cases they will need to take additional steps prior to beginning Module II.

1. Determine the Concentration of the Original Solution (Experimental Condition 1).

Many pollutants will be solid in their purest forms. A 1000 ppm solution can be created by combining 1 mg of the solid pollutant with 1 ml of water. If the pollutant cannot be expressed as a concentration in mg/ml – as with many detergents – calculate concentration based on volume keeping in mind that 1000 ppm = 0.1%.

If possible research the pollutant to find a range of concentrations that will result in observable plant growth differences. The starting solution will be the highest concentration. This solution will be diluted to make experimental conditions 2 and 3.

Keep in mind that combining the pollutant solutions with plant growth media will dilute the final concentration of your pollutant. For example, the five provided solutions are provided at a starting concentration of 15 mg/ml (15,000 ppm) or 150 mg/ml (150,000 ppm). However, their final concentrations in the petri dishes are 1,000 ppm or 10,000 ppm.

2. Prepare Experimental Condition 2 and Experimental Condition 3.

You will use the general equation:

$$C_1 \times V_1 = C_2 \times V_2$$

C_1 = Initial concentration
V_1 = Initial volume
C_2 = Final concentration
V_2 = Final volume

To prepare experimental conditions 2 and 3 you will rearrange the equation to solve for V_1.

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

C_2 = Desired concentration for the experimental condition (see Module I).
V_2 = 1600 µL
C_1 = The starting solution concentration discovered in step 1.

Subtract the initial volume (V_1) from the final volume (V_2) to determine the amount of water necessary to create the dilution. Add the water to the concentrated pollutant and mix thoroughly.

3. Determine the final pollutant concentration when each experimental condition is combined with plant growth media for the three petri dishes.

Use the general equation shown in step 2 but solve for C_2.

$$C_2 = \frac{C_1 \times V_1}{V_2}$$

C_1 = The experimental condition concentration calculated in step 2.
V_1 = The volume of experimental condition solution added to the plant growth media (we suggest 1000 µL).
V_2 = The total volume of plant growth media and experimental condition solution added to the petri dish (we suggest 15,000 µL).

Appendix C
Representative Experimental Results

Below are experimental results for five seeds using vertical plates and measuring root length, stem length, plant wet weight, and germination success for the other provided pollutants. Please note that these are representative examples from the EDVOTEK® laboratory; student results may vary.

Pollutant: Zinc	**Root Length Average**	**Root Length Std. Dev.**	**Stem Length Average**	**Stem Length Std. Dev.**	**Plant Wet Weight Average**	**Plant Wet Weight Std. Dev.**	**Germination Success**
Control	63.4 mm	40.8 mm	31.8 mm	18.3 mm	100.0 mg	62.5 mg	80%
100 ppm	50.6 mm	16.2 mm	31.6 mm	16.56 mm	78.3 mg	34.0 mg	100%
1,000 ppm	14.8 mm	5.4 mm	7.4 mm	2.41 mm	34.2 mg	14.3 mg	100%
10,000 ppm	6.6 mm	2.4 mm	2.6 mm	2.2 mm	20.5 mg	9.3 mg	100%
Pollutant: Nickel	**Root Length Average**	**Root Length Std. Dev.**	**Stem Length Average**	**Stem Length Std. Dev.**	**Plant Wet Weight Average**	**Plant Wet Weight Std. Dev.**	**Germination Success**
Control	49.2 mm	30.3 mm	31.4 mm	22.6 mm	62.5 mg	41.7 mg	100%
10 ppm	19.2 mm	7.8 mm	20.4 mm	3.7 mm	59.8 mg	17.1 mg	100%
100 ppm	12.0 mm	5.0 mm	6.8 mm	4.1 mm	24.9 mg	12.5 mg	100%
1,000 ppm	12.2 mm	4.8 mm	5.4 mm	4.3 mm	24.0 mg	13.3 mg	100%
Pollutant: Salt	**Root Length Average**	**Root Length Std. Dev.**	**Stem Length Average**	**Stem Length Std. Dev.**	**Plant Wet Weight Average**	**Plant Wet Weight Std. Dev.**	**Germination Success**
Control	52.6 mm	25.8 mm	38.4 mm	15.6 mm	84.6 mg	38.4 mg	100%
100 ppm	18.8 mm	12.8 mm	18.4 mm	14.4 mm	57.6 mg	41.0 mg	80%
1,000 ppm	13.6 mm	9.8 mm	11.0 mm	6.52 mm	31.1 mg	20.8 mg	100%
10,000 ppm	2.2 mm	4.9 mm	0.6 mm	1.3 mm	12.9 mg	32.3 mg	20%
Pollutant: Acid Rain	**Root Length Average**	**Root Length Std. Dev.**	**Stem Length Average**	**Stem Length Std. Dev.**	**Plant Wet Weight Average**	**Plant Wet Weight Std. Dev.**	**Germination Success**
Control (pH 5.0)	58.6 mm	18.8 mm	31.6 mm	18.8 mm	83.9 mg	44.1 mg	100%
pH 4.5	20.0 mm	18.6 mm	20 mm	19.6 mm	54.5 mg	58.1 mg	60%
pH 3.7	29.4 mm	19.4 mm	29.4 mm	19.4 mm	60.1 mg	40.0 mg	100%
pH 2.3	0 mm	0 mm	0 mm	0 mm	13.3 mg	1.9 mg	0%