LAB MODULE: AN INVESTIGATIVE ZEBRAFISH TOXICITY ASSAY
THE TOXICITY ASSAY

CONCEPTS: STUDENTS WILL UNDERSTAND THAT
1. Toxicology is the study of harmful effects of chemicals on living things.
2. Toxicology involves interactions between biology, chemistry, environmental science and human health.
3. Dose/response bioassays provide a measure of toxicity.
4. Chemical risks are relative, and every chemical is toxic at a high-enough dose.
5. There is no such thing as "zero risk. Setting environmental standards requires both scientific data and human judgment to determine what level of risk is acceptable to society.
6. Science is multidisciplinary and related to societal concerns.
7. Clear presentation of research results is an integral part of the scientific process.
8. Scientists work both individually and collaboratively, reviewing each other's work to provide feedback on experimental design and interpretation of results.
9. Scientific understandings are tentative, subject to change with new discoveries.

PERFORMANCE SKILLS: STUDENTS WILL BE ABLE TO:
10. Conduct scientific research, starting with well-defined protocols and progressing to open-ended research projects.
11. Work collaboratively to design experiments, interpret results, and critically analyze ideas and conclusions.
12. Define a toxicological research question, then plan and carry out an experiment to address this question using bioassays with one or more types of organism.
13. Analyze data and draw conclusions about toxicity and risk.
14. Identify sources of variability in data, including potential sources of bias.
15. Write a concise and accurate summary of methods, results, and conclusions.
16. Properly use basic laboratory equipment including a balance, micropipettors and a pH meter.
17. Identify the parts of a properly designed experiment.

REFERENCES
- Basics on Bioassays and experimental design: (http://ei.cornell.edu/toxicology/bioassays/)
- TOXNET - Databases on toxicology, hazardous chemicals, environmental health, and toxic releases. (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)
- Comparative Toxicogenomics Database (CTD: http://ctd.mdibl.org/
- Creating an Effective Poster Presentations: An Effective Poster (http://www.ncsu.edu/project/posters/NewSite/)
EXERCISES:
1. Part I. Learn about toxicity assays - Use references and web sites to obtain background information about a chemical to test.
2. Part II. Working with zebrafish embryos - Determine the LD₅₀ of a toxic chemical using zebrafish as a model organism. (To be completed over multiple weeks.)
3. Part III. Learn to use basic laboratory equipment for measuring mass, volume and pH.

MATERIALS AVAILABLE IN LAB:
Zebralish Assay
petri dishes
E3 media
> 24 hour embryos
various chemicals
distilled water

TOXICITY ASSAY (BIOASSAY) (A Brief Introduction)
You will be determining the LD₅₀ of a toxic chemical of your choice. In order to do this you must first determine a chemical to test and its availability. Research the basis of its toxicity, then design and complete the determination of the chemicals LD₅₀ on 24 - 72 hour zebrafish embryos. During the next several weeks these experiments will be the focus of your laboratory work and will require time outside the lab. Plan wisely. At the end of this module each group will give a poster presentation as scheduled in the syllabus.
TOXICITY ASSAYS: DETAILS

Part I. Each group will begin this bioassay module by looking up background information on a chemical compound either chosen or assigned. Use the web sites suggested on the course laboratory web page and other resources to look up information on your chemical including its chemical structure, physical characteristics, and the major mechanism(s) of toxicity on living organisms. A required objective for this module on bioassays will be to develop a dose-response curve in order to determine the LD50 of your compound on 24 hour zebrafish embryos (age of embryos may vary depending on availability). The LD50 is defined as the dose that causes mortality in 50% of the organisms tested estimated by graphical or computational means. The results of this bioassay module will be in the form of a poster presentation that will include the following sections: title, abstract, background information, materials and methods, results, conclusions, and references. Suggestions for creating your poster may be found at: http://www.ncsu.edu/project/posters/NewSite/.

Where to start?
1. Identify the chemical to study.
2. Go to: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM (shown below)

2. Type in your chemical (example shows results after entering cadmium)
3. Click on Full record.
4. Scroll down until the toxicity data appears

5. Record the LD$_{50}$ of your chemical as it was determined in other organisms. You will use this information to determine the starting concentrations in which to begin your assay. In order to make calculations less burdensome several assumptions will be made. First, we will assume that the concentration of the compound in the water around the embryos will be equal to the concentration inside the chorions (outside shell around the zebrafish embryos) and the cells of embryos. This may not be true since the chorions may exclude certain compounds and the zebrafish may contain specific ABC type transporters that function to pump toxic substances out of (or into) the body tissues leading to an artificially high or low LD$_{50}$ value. As an example, some toxicity values for cadmium compounds are listed below.

- mouse (intraperitoneal) 5.7 mg/kg
- mouse (oral) 890 mg/kg
- rabbit (intravenous) 5 mg/kg
- rabbit (subcutaneous) 6 mg/kg

Notice that the LD$_{50}$ depend on the route of entry into the body of the organism being tested. The zebrafish embryos may be more or less sensitive to toxins than the other organisms tested so choose the lowest value as a starting point. For example from the data above the intravenous route tested in rabbits (5 mg/kg) would be your beginning target value.

6. Convert the units from w/w (mg/kg) into w/v (mg/L). (Remember 1 g H$_2$O = 1 ml H$_2$O or 1 kg H$_2$O = 1 L H$_2$O). The zebrafish embryos will be in a fluid environment called embryo medium, E3. This means that a value of 5 mg/kg would be equal to 5 mg/L or after dividing both the numerator and denominator by 1000 = 5 ug/mL. Because you will be working with toxic substances, you will need to make a small volume of the substance at a dose high enough to measure accurately. For example, if the LD$_{50}$ is around 5 ug/mL set up a range of concentrations below and above this value (i.e. 0.5 ug/mL to 10 ug/mL)
7. At this time it helps to know how the bioassays will be performed. (see Part II below, which will be demonstrated in lab)

8. Making your stock and test solutions. Since it would be difficult to accurately weigh such small amounts of chemical, you must first make a concentrated stock solution then dilute this stock to get each final working solution. (Note: you will only need 5 to 10 mL of each concentration to be tested). An example is given below however do not make any solutions until your plans are approved by the instructor.

Example: Making a stock to test toxicity of cadmium at concentrations ranging from 0.5 ug/mL to 10 ug/mL.

1. Unless you are dissolving a tablet or capsule with a predetermined amount of active ingredient, assume you can not accurately weigh less than 100 mg of your test chemical. Depending on the degree of solubility, dissolve in as little water as possible. In this example, 100 mg of cadmium chloride is dissolved in 10 mL of ultrapure water. **You must label all containers with the contents including the concentration, date, and your last name. All unlabelled containers will be thrown away. Do not place anything in the laboratory drawers. All groups will be assigned a space to store experimental supplies.**

100 mg/10 mL = 10 mg/mL. You will not need more than 5 mL of each concentration to be tested therefore the following dilutions (using \( \text{C1V1} = \text{C2V2} \)) can be set up. Begin by making a 1/10 (1:9) dilution of your stock (1 ml stock in 9 mls water = 1 mg/ml = 1000 ug/mL)

**Tube 1:** control (E 3 alone)

**Tube 2:** (0.5 ug/ml) (5 mls) = (x mls)(1,000 ug/ml) 2.5 uls 1x stock in 5 mls embryo water

**Tube 3:** (1.0 ug/ml) (5 mls) = (x mls)(1,000 ug/ml) 5.0 uls 1x stock in 5 mls embryo water.

Follow this pattern to make the rest of your dilutions. It is important to note that each chemical may have its own unique features that will require modifications to this protocol. For instance if your substance is a pesticide that has a large percentage of inert ingredients mixed with the active ingredient your will need to adjust for these inert ingredients. All groups are required to set up an appointment with me to go over your calculations before you begin.

**Part II. Information on bioassay procedures**

Zebrafish are keep under strict protocols of light/dark cycle do not enter the prep room during the dark cycle!

The instructor will try to have newly fertilized embryos available at least 2 times/week. These embryos will be dirty and must be individually placed in clean E3 for 24 hours before being used in an assay. Planning ahead is required. Take only the number of embryos you need and return embryos you do not use so that others may use them. (The maximum number of embryos to be used per bioassay is 50 embryos.)

**Instructions for handling embryos and setting up assays will be provided during lab.**
BIOASSAY PROCEDURE

1. All assays will be done using E3 embryo medium (See prep room door for details). A 50X stock solution is diluted by placing 20 mls of stock in a 1 liter container and q.s. to 1 liter with dH$_2$O.

2. Assays will be done in 96 well plates with one embryo added to each well. The different concentration of your compound to be tested will be placed in rows A through H. Use 10 embryos at each concentration to be tested.

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3. Determine the concentrations of the chemical to be tested. Only 5 concentrations can be tested per 24 hours. All assays must include a control which tests E3 media alone.

4. Begin setting up your assay by placing one healthy 24 hour embryo in each well (1 through 10). Due to different rates of development you must look at each embryo under a dissecting microscope to ensure it is alive and looks like a 24 hour embryo should look like!

5. Use a p200 micropipettor and carefully remove most of the fluid from around each embryo in Row A. Replace fluid with 200 uls of E3. Work carefully and quickly, do not allow embryos to dry out.

6. Repeat this process for rows B through H by replacing the fluid with 200 uls if your test solution.

7. After 24 hours score and record each embryo as alive or dead. Graph your results as % dead vs. concentration. If all your embryos are all dead or all alive you must reevaluate your range of test concentrations and retest.

8. Graph % dead vs. conc using a scatter plot.

9. Determine the equation of the line produced to determine the concentration at which 50% died.

10. Your final graph and LD$_{50}$ determination must be the average of three independent experiments and include error bars representing the standard deviation.
Additional questions to answer:

1. Did at least 80% of the zebrafish embryos in the control sample survive? If not, what would you recommend doing differently next time to try to get a better survival rate?

2. Did the rate of zebrafish embryo survival respond in a predictable way to concentration? Describe the trends you observed.

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

4. What LD_{50} would you estimate for your experiments with zebrafish embryos? If it is impossible to estimate the LD_{50} from your data, please explain why.

5. What can you conclude about the toxicity of the substance you tested? Is this what you expected?

6. Think about whether any of the embryos might have died for reasons other than poisoning by the chemical you tested. What other factors do you think might possibly have killed some of them?

7. If other students carried out a dos/response experiment using the same chemical (or different salt form), did their data follow the same trend as yours?

8. Based on this experiment, would you say zebrafish embryos would provide a useful toxicity assay organism for water samples from the environment? Why or why not?

9. If you were going to repeat this experiment, what would you do differently? How might you improve the experimental design to reduce variability of your data or lead to more reliable results?
From: Kimmel et al. Stages of embryonic development of the zebrafish
Dev. Dyn. 203:253-310, 1995