

*A brine shrimp bioassay to measure toxicity and remediation
of chemicals*

Supplemental Materials

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A brine shrimp bioassay to measure toxicity and remediation of chemicals

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I. Introduction

This article describes a laboratory project which uses a bioassay on brine shrimp to measure LD-50 values for a variety of insecticides. This project was the centerpiece of a five-week unit on toxicology and chemical remediation in my non-science majors course, *Chemistry and Public Policy*. The 22 students in the class were first-year liberal arts majors, most with fairly solid high school chemistry backgrounds. Two 3-hour lab periods are required, the first for toxicity testing, and the second for remediation and retesting.

Any chemical that can be dissolved or dispersed into water can be studied in this bioassay: plant extracts or other natural products, real or simulated "hazardous waste," metal ions, agricultural chemicals, food additives, home cleaning products, or pharmaceuticals. Although the bioassay is simple enough to use with students who have little prior lab experience, it also could be integrated into an environmental, organic, medicinal, or biochemistry lab course.

This set of labs helps students learn how toxicity is measured and how harmful molecules can be decomposed to harmless products. Ethical issues are introduced in a discussion of animal testing. Students learn to search the literature and predict reactivity from structure; this aspect of the lab could be emphasized more for students with more chemistry knowledge.

The bioassay and remediation procedures described in the supplementary material could be used as straight labs or "set up" as a multi-week project. In one such project, students were given a letter from the owner of a dog-grooming salon, worried that the insecticide products she uses in the salon are leaching into a nearby stream. She asks the students to test the flea products to determine their toxicity to aquatic life, and to devise some method that she could use to make the wastewater less toxic before she pours it down the drain.

The students were then divided into groups of 3 or 4 and given an insecticide product. The students measured the LD-50 value of their insecticide on brine shrimp; all seven groups were able to complete this lab in a three-hour lab period. Over the next two weeks, the groups researched the chemicals present in their insecticide product. During this time, they studied basic organic chemistry. Based on the structure and reactivity of the main active ingredient, each group decided on a method of remediation to test in lab. In the second lab period, the remediation procedures were carried out and the remediated material was assayed on brine shrimp. The project culminated in a poster session at which each group presented its results.

II. Experimental

A. The Bioassay

There are several published whole-animal bioassays for assessment of chemical toxicity.ⁱ Bacteria are commonly used for quantitative tests, and there are some procedures for qualitative tests on *Blephisma* (a one-celled pond organism) and *Daphnia* (water fleas).

Brine shrimp have been used as a “benchtop bioassay” for the discovery and purification of bioactive natural products,ⁱⁱ and they are an excellent choice for elementary toxicity investigations of consumer products. Brine shrimp, *Artemia* species, also known as sea monkeys, are marine invertebrates about 1 mm in size. Freeze-dried cysts are readily available at aquarium stores. The cysts last for several years and can be hatched without special equipment (directions are given in the supplementary materials). Toxicity testing is carried out by adding different doses of material to small numbers of the shrimp; the entire dose-response curve for a substance can be generated in a three hour lab period. The supplementary material gives a general procedure for this bioassay.

The assay does involve killing brine shrimp. There is a reasonable controversy over use of animals for such purposes, and some people have ethical or religious objections to killing even “lower” organisms. Although a few of my students had formed opinions about the Draize test or about dissecting frogs in high school, most had not thought much about the ethics of what they were being asked to do in this lab. Before the experiment, students investigated a variety of pro- and con-animal testing web sites.ⁱⁱⁱ This was followed up by a pre-lab discussion about animal testing and alternatives, and which species the students would be willing to use as experimental animals under different circumstances. It was particularly informative to pass around some actual

brine shrimp at this discussion--some of the students had rather overblown expectations derived from sea monkey ads in comic books!

B. Assaying the toxicity of insecticides:

Students examined seven products, listed in Table 1. These products all contained insecticide and a variety of inert ingredients, such as solvent or detergents. The students measured all concentrations in terms of the formulated product, rather than the active ingredient. Although “inert” ingredients such as toluene or detergents are less toxic than the actual insecticides, they are generally present in higher concentrations and hence could be responsible for some of the toxicity of these products to brine shrimp.

Each product was assayed by 3 or 4 students. They measured the dose required to kill half of a group of shrimp (LD-50) and the dose required to cause sub-lethal behavioral effects (slow swimming rate) in half of a group of shrimp (LE-50). The values varied within an order of magnitude due to a variety of factors: poor pipetting technique, mixing the tubes too vigorously (this can literally shake the shrimp to death and should be suspected if a student gets moderate levels of mortality regardless of dose), difficulty in counting the shrimp or observing sub-lethal effects, and errors in constructing smooth curves and interpolating the LD-50 and LE-50 values, which are on a log scale. Despite the range of error, some products are clearly more toxic to brine shrimp than others; several of the products were not lethal even at the highest doses tested. Contrary to the students' expectations, “natural” insecticides such as α -limonene and pyrethrins were no less toxic than “artificial” ones like diazinon.

Table 1. Toxicity data for various flea products on brine shrimp. For each product, the range of LD-50 and LE-50 values obtained by each group of 3-4 students is shown.

Group	Insecticide product	Active Ingredients	LD-50 (ppm)	LE-50 (ppm)
I	Zodiac Pyrethrin Dip	Pyrethrins 0.3% Piperonyl butoxide, tech. 3.00%	40-418	1-32
II	Zodiac Limonene Dip	d-Limonene 78.2%	28-100	1-3

III	Hi-Yield Diazinon 4E spray	Diazinon 47.5%	4-63	4-9.5
IV	Releve Flea and Tick Conditioning Shampoo for Dogs	d- <i>trans</i> -Allethrin 0.10% N-Octyl bicycloheptene dicarboximide 0.15%	>1670	16
V	Flea Stop Plus Shampoo	Linalool 3.7%	700-900	0.6-2.4
VI	Tomlyn Flea, Tick, Lice Shampoo	Pyrethrins 0.054% Piperonyl butoxide, tech. 0.108% N-Octyl bicycloheptene dicarboximide 0.18%	1400- >1670	10-25
VII	Flea Stop 42-Day Dursban Spray	Chlorpyrifos 0.50%	>1670	418-1000

C. Remediation of insecticides:

Students were given procedures for four methods of remediation: oxidation/hydrolysis with bleach, Cu(II)-catalyzed hydrolysis, photolysis, and adsorption onto activated carbon (these are described in the supplementary material). An alternate option would be to use a published wastewater treatment procedure.^{iv} In order to choose an effective procedure, students need to know how to read Lewis structures and shorthand organic structures, use the structures to predict molecular polarity and solubility, and recognize simple functional groups such as esters, phosphate esters, and hydrocarbons.

Table 2. Equipment required

Brine shrimp bioassay	Brine shrimp cysts, sea salts (Aquarium store) Air bubbler (Aquarium store or can use compressed air)
Remediation procedures	Sunlight or UV lamp (borrow from Organic lab), cation exchange resin e.g. Bio-Rex 70

The instructor met with each group of students for 20 minutes to discuss their choice of method, what they planned to vary in the method, appropriate control experiments, and to make sure they understood the experimental procedure that each one would follow in lab. In preparation for the meeting, each student was required to find and interpret toxicity information about their pesticide using the Merck index, RTECS, and on-line MSDS sheets. They also read articles on pesticides and decontamination of chemical weapons.^v These meetings were essential for the unstructured lab which followed.

Group results were reported in a poster session. Even if a product's LD-50 value was larger than the highest concentration assayed, the students were still able to measure changes in the LE-50 value before and after remediation. Figure 1 shows data on the remediation of a pyrethrin-containing dog shampoo. The shampoo caused lethargic swimming in brine shrimp at a dose of 16 ppm. After treatment with activated charcoal, the shampoo did not cause any observable effect on brine shrimp even at 1670 ppm, the highest dose tested.

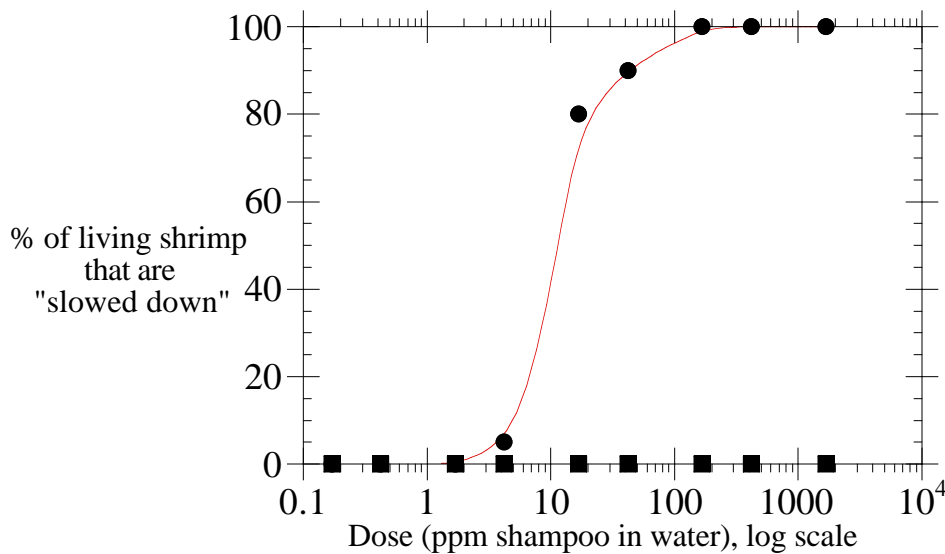


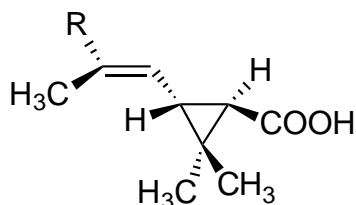
Figure 1. Student data for Relevee Flea and Tick Conditioning Shampoo® before and after remediation. The untreated shampoo at a dose of about 16 ppm causes sub-lethal effects (lethargic motion) in brine shrimp. (circles). Treatment with activated carbon completely eliminates this effect, even at doses of 1670 ppm (squares).

III. Acknowledgements: Thanks to Prof. M. Miller, who suggested we try the brine shrimp bioassay, and to Mathew Snyder, who spent many hours testing the lab procedures and rounding up odd supplies. The development of this project was supported by the Camille and Henry Dreyfus Foundation under SG-98014.

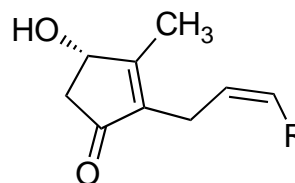
I. Chemical background (flea killers)

Most commercial flea killers fall into one of three categories: natural or artificial pyrethrins, often with a sensitizer such as piperonyl butoxide or N-octyl bicycloheptene dicarboximide, organophosphonates, and terpenoid natural products such as linalool or limonene.

Natural pyrethrins^{vi} are extracted from chrysanthemum flowers, where they make up 0.3-2% of the dry weight of the flower. The flowers are extracted with a combination of methanol and kerosene to give crude pyrethrum, which contains about 25% pyrethrins, mostly Pyrethrin I and II. Natural pyrethrins are esters of the acids **1** or **2**, and the β -hydroxy ketones **3**, **4**, or **5**. The ester linkage is susceptible to hydrolysis under acidic or basic conditions, and the allyl cyclopropane ring is sensitive to sunlight and air oxidation. The relative reactivity of pyrethrins can be an advantage, because the insecticide is not persistent in the environment.



- 1** R=CH₃, (+)-*trans*-chrysanthemic acid
2 R=COOCH₃, (+)-*trans*-pyrethoic acid



- 3** R=CH₃, (+)-jasmolone
4 R=CH₂CH₃, (+)-cinerolone
5 R=CH=CH₂, (+)-pyrethrolone

Figure 1. Building blocks of natural pyrethrins

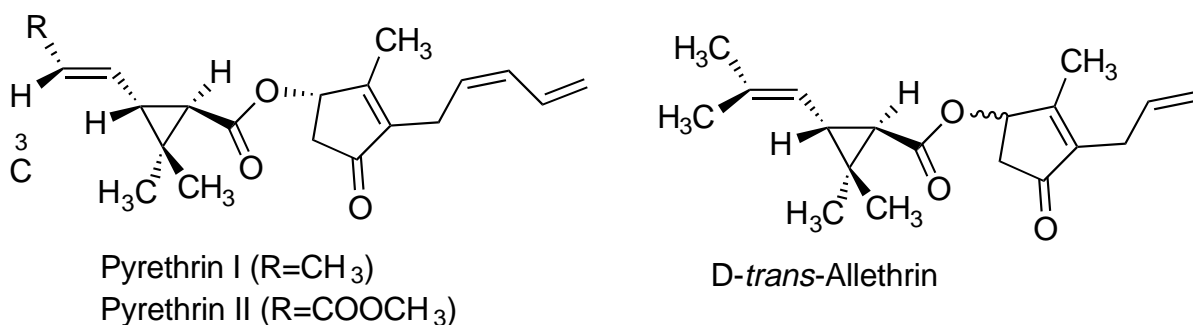


Figure 2. Natural and artificial pyrethrins

Artificial pyrethrins, including allethrin, fenvalerate, permethrin, and decamethrin, are used on field crops like cotton and cabbage; they break down rapidly in the soil, but have good stability on leaf surfaces. The pyrethrins are all optically active, and in most cases, biological activity is centered in a single isomer; there is thus a significant drive to produce these bulk chemicals in optically pure form.

The pyrethroids interfere with nerve transmission in insects, which causes “knock-down” of flying insects. The biochemical targets of the pyrethrins include voltage- and γ -aminobutyric acid-gated ion channels; inactivation of these channels blocks the propagation of electrical signals in neurons. The main biochemical route of pyrethrin detoxification is oxidation mediated by monooxygenase enzymes such as cytochrome P-450.^{vii} Synergistic agents such as piperonyl butoxide or N-octyl bicycloheptene dicarboximide are often added to pyrethrin insecticides; the synergists inhibit monooxygenases, and thus allow lower doses of the pyrethrin to be effective. Many species of insects have developed resistance to pyrethrins, often through mutations giving more efficient monooxygenases, and development of new synthetic pyrethrins remains an active area of research.

Organic phosphates,^{viii} such as the insecticides diazinon and chlorpyrifos shown in Figure 3, are inhibitors of the enzyme acetylcholinesterase. Organic phosphate esters and thioesters mimic the tetrahedral transition state involved in the hydrolysis of acetylcholine, and thus are able to bind inside the active site of acetylcholinesterase. An active site serine residue displaces one of the leaving groups on the phosphorous, producing a phosphorylated enzyme which is unable to accommodate acetylcholine in the active site. Phosphorylation can generally be reversed by hydrolysis, but a sufficient dose of an insecticide can inactivate most of the acetylcholinesterase molecules in an organism for a fatal length of time. The neurotransmitter acetylcholine builds up, resulting in convulsive muscle contraction and ultimately death. These chemicals are closely related to human nerve gases both structurally and synthetically; one of the practical challenges for the implementation of the Chemical Weapons Convention^{ix} is the ease of converting a factory which makes organophosphorous pesticides, into one which makes nerve gas.

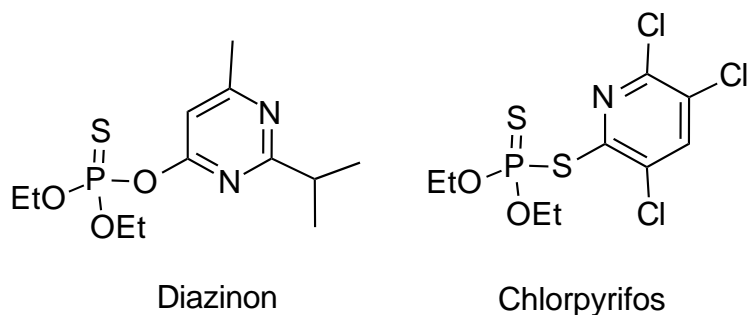
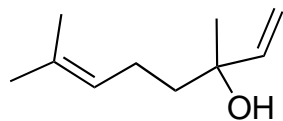


Figure 3. Common organophosphorous insecticides.

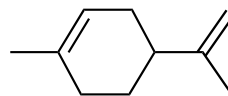
Most organophosphate insecticides intended for use on pets are phosphate thioesters, because the mammalian toxicity of these chemicals is significantly lower than the insect toxicity. In insects, the same oxidative enzymes which help to detoxify the pyrethrins are capable of transforming phosphate thioesters into more toxic phosphate esters. This biotransformation is much slower in mammals, so Fido can tolerate a dose of chlorpyrifos which is fatal to his fleas. Insects which develop a resistance to pyrethrins through mutation in their oxidative enzymes often have a concomitant sensitivity to phosphate thioester insecticides due to this bioactivation process, which means that the farmer can alternate use of pyrethrins and organophosphorous pesticides for better control of insect pests. In other cases, however, insect populations develop resistance to organic phosphates through mutations to the target acetylcholinesterase or an increase in the rate of conjugation of organophosphate esters to glutathione. There are now many species of insects which show multiple resistances to different classes of insecticides.

Organophosphates are quite susceptible to hydrolysis, and thus they do not persist in the environment. Reddy *et al.* studied the decomposition of diazinon in compost heaps; within 18 days, nearly 99% of the diazinon had been hydrolyzed.^x Hastening the degradation of phosphate esters and thioesters has been studied quite a bit because of the threat of military or terrorist use of nerve gases, and the widespread use of organophosphorous pesticides. Hydrolysis of phosphate thioesters can be accelerated in the laboratory by a variety of catalysts, including oxidants, acid or base, and Lewis acids such as metal cations.^{xi-xii}

Many essential oils contain terpenoid chemicals which function as insect repellants. For example, citronella extract is widely used to discourage mosquitoes and other flying insects. Limonene, a compound found in all citrus oil, and linalool, a compound found in oil of linaloe, cinnamon, and bergamot, are in this category. We found the toxicity of limonene to be as high as that of many synthetic insecticides, so it must be more than a repellant. I could not locate information on the biochemical target of limonene or linalool. Both limonene and linalool are quite hydrophobic; hydrophobic compounds are able to penetrate the waxy layer on insect cuticle and dissolve in cell membranes. Effective remediation could be obtained by adsorption on activated carbon or even on dirt.



Linalool



Limonene

Figure 4. Two common terpenoid insect repellants.

II. Experimental

A. Care and feeding of Brine Shrimp (*Artemia franciscana*)

Artificial sea water is made by adding 37 g/L of sea salts such as Instant Ocean[®] (available at any aquarium store) to distilled or deionized water. The sea water can be stored at 4 °C for several weeks.

Brine shrimp cysts are available at most pet shops which sell tropical fish. One source, O.S. I. Pro 100 Artemia Cysts, Ocean Star International, Inc, Snowville, UT 84336, supplies about 100 g of vacuum-packed cysts for \$4-5. To hatch the brine shrimp, a large beaker is equipped with an air bubbler (this could be an aquarium pump with an airstone or just a pipet connected to a source of compressed air) and filled with sea water. Brine shrimp cysts (about 0.4 g/L of sea water) are added; the aeration should keep the cysts moving about in the water. Brine shrimp will hatch in 24-36 hours at 25 °C. With normal hatching rates, these conditions should yield at least 20 live brine shrimp/ml of water. The shrimp should be used between 2-4 days after hatching (newly hatched shrimp are so small they are hard to count).

B. LD-50 tests of consumer products

It is convenient to conduct the tests in 13 mm culture tubes or test tubes. Ten clean culture tubes are obtained. 5.00 ml of sea water is put into one tube and the 5.0 ml level is marked; the sea water is poured out and the 5.0 ml line is transferred to all the tubes. About 10 brine shrimp (in about 0.5 ml of sea water) are added to each tube, and then the tubes are filled to the 5.0 ml line with sea water.

The material to be tested is serially diluted with sea water to make 100 x (1 g product per 100 ml), 1,000 x, 10,000 x, 100,000 x, and 1,000,000 x dilutions. The test tubes are dosed as shown in Table 1, with the low dosages being prepared first. As each tube is prepared, the time of dosing should be recorded. Adequate accuracy can be obtained with disposable graduated transfer pipets (e.g. Fischer catalog #13-711-9A).

Table 1. Dosage chart.

Test tube	Dosage	Concentration of product*
1	1.0 ml sea water	0
2	1.0 ml of 1,000,000x	0.17 ppm
3	0.25 ml of 100,000x, 0.75 ml sea water	0.42 ppm
4	1.0 ml of 100,000x	1.67 ppm
5	0.25 ml of 10,000x, 0.75 ml sea water	4.2 ppm
6	1.0 ml of 10,000x	16.7 ppm
7	0.25 ml of 1,000x, 0.75 ml sea water	42 ppm
8	1.0 ml of 1,000x	167 ppm
9	0.25 ml of 100x, 0.75 ml sea water	418 ppm
10	1.0 ml of 100x	1670 ppm

*This is the bulk concentration of the product; note that the concentration of the active ingredient(s) may be much lower.

Acute responses can be observed at 1/2 hour or 1 hour after dosing, and chronic effects can be observed at 24 or 48 hours. The brine shrimp are best seen against a dark background with good light from the side of the tube. Normally, most of the shrimp swim vigorously; their swimming rate at room temperature is several beats per second, and the shrimp swim towards light (phototaxis). Shrimp that lie motionless at the bottom of the tube are dead. The most common sublethal effect is impairment of swimming rate. For each tube, students should note the fraction of shrimp that are dead and the fraction of the living shrimp showing lethargic swimming.

LD-50 values are obtained from this data by plotting the fraction of surviving shrimp against the dosage in each test tube, plotted to a logarithmic scale (see Figure 2). The ppm value corresponding to 50% survival rate is measured from these curves. In Figure 2 above, the LD-50 value is about 100 ppm for the bulk flea dip. LE-50 values for sublethal effects can be obtained by a similar analysis of sublethal effects data.

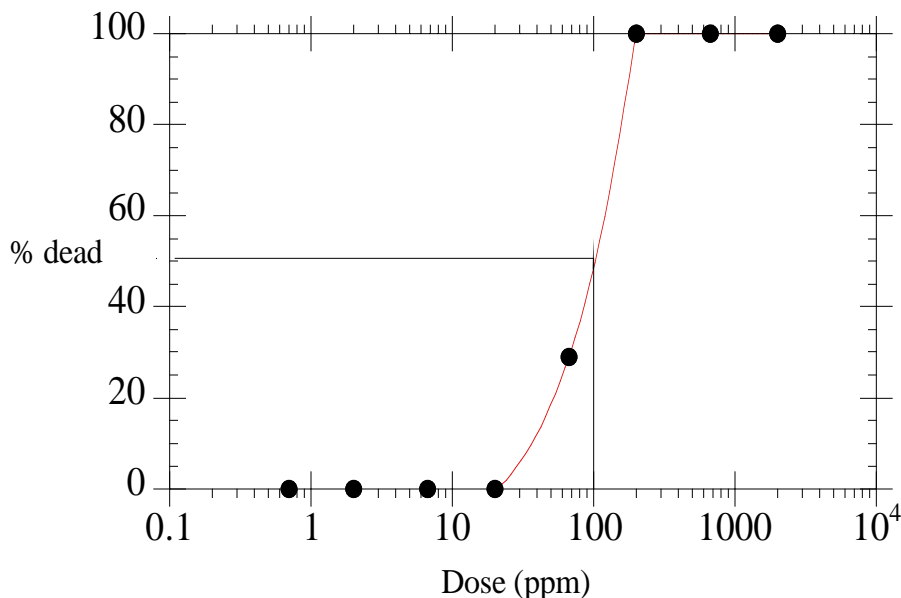


Figure 2. Toxicity of Zodiac Pyrethrin Dip[®] to brine shrimp at $t = 1$ hour (student data). The LD-50 is about 100 ppm.

C. Remediation procedures for consumer products

1) Basic bleach (oxidation and hydrolysis)

In a 125 ml conical flask are mixed 25 ml of 100 X diluted material, 0.5 g K_2CO_3 , and 5.0 ml of commercial bleach (e.g. Chlorox, 5.25% sodium hypochlorite). The reaction mixture is stirred magnetically for 20 minutes. The remaining bleach is neutralized by adding small amounts (about .1 g at a time) of sodium metabisulfite. After each addition of metabisulfate, the solution is tested for bleach with starch/iodine paper; if the paper turns blue, more metabisulfate is added.

2) Copper-catalyzed hydrolysis (hydrolysis)

In a 125 ml conical flask are mixed 25 ml of 100X diluted material and 0.25 g copper sulphate ($CuSO_4 \cdot 5H_2O$). This mixture is heated to $80^\circ C$ for 15 minutes. Excess copper is removed by adding 3.0 g of cation-exchange resin (e.g. Bio-Rex 70, an acrylic resin with sodium carboxylate side chains), stirring for 5 minutes (the resin will turn light blue) and filtering off the resin.

3) UV treatment (photodegradation)

25 ml of 100 X diluted material is placed in a petri dish or recrystallization dish and exposed to bright sunlight or a UV lamp for 40 minutes.

4) Activated carbon (adsorption)

25 ml of 100 X diluted material and 2.5 g of activated carbon are stirred at room temperature for 20 minutes, then the liquid is filtered through filter paper.

III. Letter from Plush Pup Grooming Saloon

Dr. Kim Bigshot
Director, Research Division
Cow Chemical Company

28 March 1998

Dear Dr. Bigshot,

I own and operate The Plush Pup, a dog-grooming shop in Indian Village, IN. An important part of my business is eradicating fleas; for this purpose I use several kinds of flea dips, sprays, and shampoos. I dispose of these insecticide solutions down the drain, and since I'm not on city sewers, they wind up in my septic system.

I am concerned that the insecticides will leach out of my septic drain field into Judy Creek, a nearby trout stream. I want you to analyze the samples of the flea products that I have sent you and tell me:

- a) Which of these products are the most harmful to aquatic life, and which are the safest?
- b) Is there a way I could treat the used insecticide solutions before I put them down the drain that would make them less toxic?

Please forward all your hourly charges to my aunt Priscilla Gotbucks, as per our previous arrangement.

Sincerely yours,

Jan Green
The Plush Pup Grooming Saloon

ML--Plase give this project to the summer interns. Have them forward their timesheets to my office for billing.

They can use the Artemia assay (procedure should be in the files) for LD-50 values, better also do LE-50's for any sub-lethal effects.

Remediation may be tricky. Hunt up procedures for activated carbon filtration, photodegradation, copper-catalyzed hydrolysis, and that basic bleach solution the military uses to decontaminate nerve gas. Tell the interns to try the procedures that look most reasonable based on the molecular structures of the insecticides. And remember to do the controls this time!!

IV. Student Handout for LD-50 Lab.

KB

Project #3: Toxicology and Remediation of Insecticides

Introduction

Once again, the summer interns of Cow Chemical Company face an exciting chemical challenge. For this project, you will work in a group of three or four students; your group assignment is given below.

In the first lab, your group will determine the **LD-50** value for a particular brand of insecticide. We will use brine shrimp (*Artemia franciscana*) as the test species to determine the effects of the insecticides on “aquatic life”. Each student in the group will carry out this experiment independently, and then the group will analyze the three sets of data. For the second lab, your group will investigate some likely **remediation** procedures for that insecticide. Each student will choose a different remediation method based on the molecular structure of your group’s insecticide. After carrying out the remediation method, the LD-50 will be remeasured to see if the remediation made the insecticide less toxic to brine shrimp.

You will write up an individual lab report and your group is responsible for producing a **poster** that reports the results of your group’s research.

Group	Members	Insecticide product
I		Zodiac Pyrethrin Dip
II		Zodiac Limonene Dip
III		Hi-Yield Diazinon 4E spray
IV		Releve Flea and Tick Conditioning Shampoo for Dogs
V		Flea Stop Plus Shampoo
VI		Tomlyn Flea, Tick, Lice Shampoo
VII		Flea Stop 42-Day Dursban Carpet Spray

Determination of LD-50 values using *Artemia franciscana* (Brine shrimp)

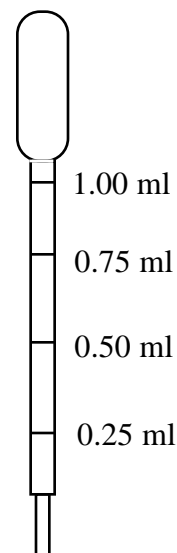
Names of people in the group:

Brand of insecticide:

1) Group tasks

Equipment needed for each group: 2L conical flask, weighing boats (located near the balance), 100 ml graduated cylinder, stirring rod. Plastic transfer pipet. 5x 250 ml conical flasks. Yellow gloves.

- a) Make up 1 L of artificial sea water by adding 37.0 g of "Instant Ocean" sea salts to 1000 ml of deionized water. Stir until all the salt is dissolved.
- b) Wash out five 250 ml conical flasks with DI water. Dry with paper towels. Label each flask with your group and "100 X", "1,000 X", "10,000 X", "100,000 X", and "1,000,000 X".
- c) Many insecticides are fairly TOXIC TO HUMANS. Whoever works with the undiluted insecticides must work in a hood and wear a pair of rubber gloves (the thick yellow ones, not the thin latex ones) and a lab coat. (Of course you are wearing your goggles too!!)
- d) Get a plastic transfer pipet and use it to measure out 1.00 ml of your flea product into the "100 X" flask. It is important that this measurement be accurate!! Throw out the pipet when you're done with it.

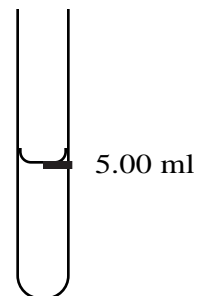


- e) Add 99.0 ml of sea water and swirl the flask for 1 minute to mix the chemicals into the sea water. This solution has now been diluted by a total of 100 X.
- f) Measure out 10.0 ml of the 100 X solution in a graduated cylinder and pour it into the flask marked "1,000 X". Rinse out the graduated cylinder with DI water. Add 90 ml of sea water to the 1000X flask and swirl for 1 minute to mix. This solution has been diluted by a factor of ten to yield a total dilution of 1,000 X.
- g) Measure out 10.0 ml of the 1,000 X solution in a graduated cylinder and pour it into the flask marked "10,000 X". Rinse out the graduated cylinder with DI water. Add 90 ml of sea water to the 10,000X flask and swirl for 1 minute to mix.
- h) Measure out 10.0 ml of the 10,000 X solution in a graduated cylinder and pour it into the flask marked "100,000 X". Rinse out the graduated cylinder with DI water. Add 90 ml of sea water to the 100,000X flask and swirl for 1 minute to mix.
- i) Measure out 10.0 ml of the 100,000 X solution in a graduated cylinder and pour it into the flask marked "1,000,000 X". Rinse out the graduated cylinder with DI water. Add 90 ml of sea water to the 1,000,000 X flask and swirl for 1 minute to mix.

2) Individual tasks

Each student will need: 5 plastic transfer pipets, test tube rack and ten test tubes, sea water, marker and labels, vial with about 10 ml of brine shrimp, watch.

a) Use your plastic pipet to measure five 1.00 ml portions of sea water into a test tube. Mark the water level at the bottom of the **meniscus** (see mark on illustration at right). Pour out the water. Check with your group to be sure all your marks are the same height from the bottom of the test tube.



b) Mark nine more test tubes at this 5.00 ml line. Label each tube with your initials and with the letters a-j.

c) Use a clean plastic pipet to put 8-10 brine shrimp in each test tube. Fill each test tube with sea water so the bottom of the meniscus comes to the 5.00 ml line.

d) Check each tube to be sure all the brine shrimp are alive and kicking. (Why?)

e) Dose the tubes according to the following table.

Test tube	Dosage	Concentration	Initial # shrimp	Time dosed
a	1.0 ml sea water (CONTROL)	0		
b	1.0 ml of 100000x	0.17 ppm		
c	0.25 ml of 100000x, 0.75 ml sea water	0.42 ppm		
d	1.0 ml of 10000x	1.67 ppm		
e	0.25 ml of 10000x, 0.75 ml sea water	4.2 ppm		
f	1.0 ml of 1000x	16.7 ppm		
g	0.25 ml of 1000x, 0.75 ml sea water	42 ppm		
h	1.0 ml of 100x	167 ppm		
i	0.25 ml of 100x, 0.75 ml sea water	418 ppm		
j	1.0 ml of 100x	1670 ppm		

f) LD-50 results should be determined between 27 and 33 minutes after a tube has been dosed. Examine each tube and count the numbers of living and dead brine shrimp. If you cannot observe any swimming or breathing motions, the shrimp counts as dead.

Test tube	Concentration	Time observed	# alive	# dead
a	0			
b	0.17 ppm			
c	0.42 ppm			
d	1.67 ppm			
e	4.2 ppm			
f	16.7 ppm			
g	42 ppm			
h	167 ppm			
i	418 ppm			
j	1670 ppm			

g) Observe each concentration level and decide whether the living shrimp are behaving differently from the control shrimp. It might be a good idea at this point to confer with your teammates to make sure you all agree on what constitutes odd behavior in brine shrimp!

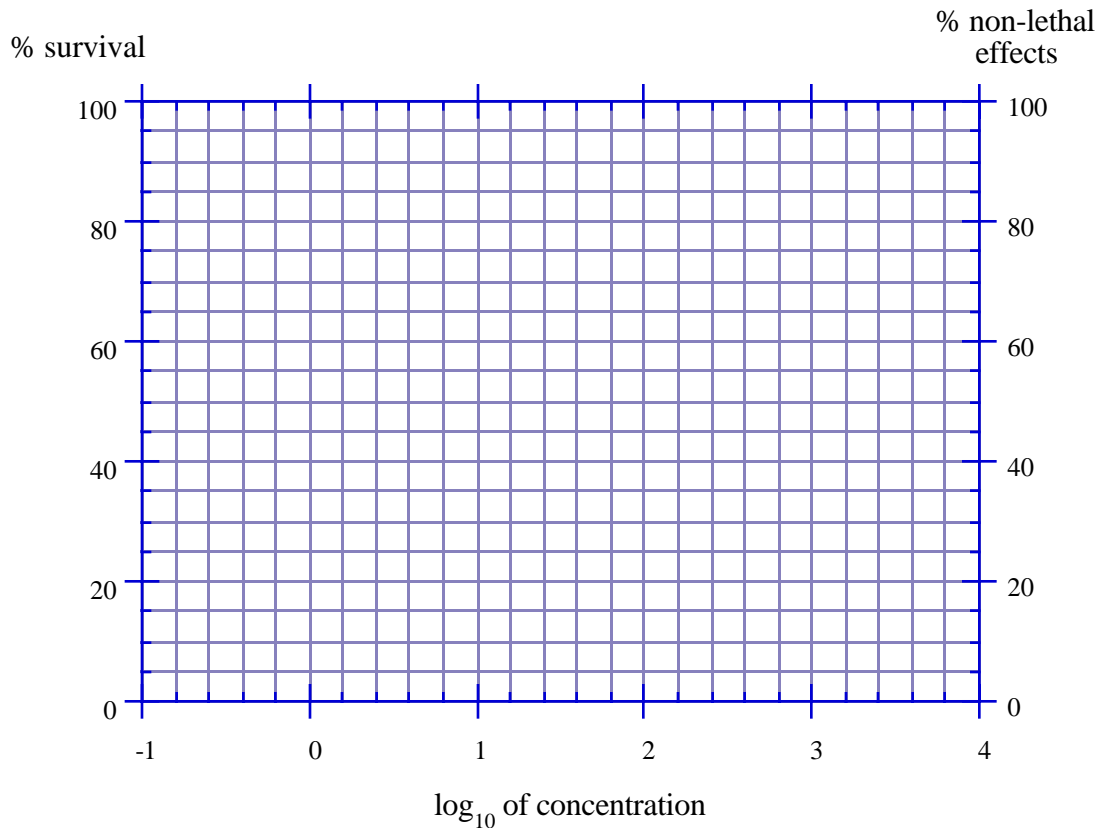
Test tube	Time observed	Describe unusual behavior of living brine shrimp	What percentage of the living shrimp are displaying this behavior?
a			
b			
c			
d			
e			
f			
g			
h			
i			
j			

h) Get out your calculator. For each concentration value, calculate the log base 10 of the concentration (for example, \log_{10} of 42 ppm would be 1.62). Then use your data from parts f and g to calculate the percentage of surviving brine shrimp and the percentage of shrimp that show sub-lethal effects.

Test tube	Concentration	\log_{10} (concentration)	% survival	% sub-lethal effects
a	0			
b	0.17 ppm			
c	0.42 ppm			
d	1.67 ppm			
e	4.2 ppm			
f	16.7 ppm			
g	42 ppm			
h	167 ppm			
i	418 ppm			
j	1670 ppm			

i) On the graph below, plot \log_{10} of the concentration as the “x” value. Use dots to show your data for % survival and crosses to show your data for % non-lethal effects.

“Connect-the-dots” to make a line graph for % survival, do the same with the crosses to get a line graph for % non-lethal effects.



j) Find the 50% survival point from your survival line graph; this corresponds to a value x of the \log_{10} of concentration. The LD-50 is ten raised to the power x . (For example, if your graph crosses the 50% level at \log_{10} of concentration = 1.83, your LD-50 is $10^{1.83} = 67.6$ ppm)

Do the same thing with your sub-lethal effect line graph to calculate the LE-50 value.

LD-50: _____
LE-50: _____

k) When all the group members have made their measurements, get together and **compare your results**. Make sure everyone did their calculations correctly. **Gross discrepancies** (LD-50 or LE-50 estimates which differ by more than a factor of 10) **mean you should repeat the experiment**.

SAVE THIS LAB SHEET--YOU WILL NEED IT TO WRITE YOUR LAB REPORT!

V. Laboratory supplies required for LD-50 lab

22 students, 7 groups

Item	Quantity
1. Disposable 1 ml transfer pipets, graduated	~ 500 (1 box)
2. 13 mm test tubes	240
3. Test tube racks (must hold at least 10 test tubes) or 500 ml beakers	22
4. 250 ml conical flasks (500 ml also OK)	35
5. 2 L conical flasks	7
6. 100 or 50 ml graduated cylinders	22
7. 10 ml graduated cylinders	7
8. Latex safety gloves (lg)	1 box
9. Acetylation vials	22
10. Labels (small)	1 roll
11. Parafilm and scissors	1 roll
12. Plastic weighing boats	36
13. Flea dips	7 bottles
14. Brine shrimp	1L batch
15. Instant Ocean	300 g
16. Yellow rubber gloves (2pr each sm, med, lg)	6 pr
17. Glass stirring rods	7

VI. Student Handout for remediation lab

On Wednesday April 15, one or more members of your group must meet with me in the Huddle. Be sure to sign up on the sheet. **The goal of this discussion is to decide on a research plan for your group to try in lab on Friday, April 17.** Sometime before this meeting, your group needs to identify the functional groups in the active ingredients of your insecticide product and decide whether oxidation, hydrolysis, photodegradation, or adsorption might help to remove or detoxify these molecules. The handouts and the lecture material will help you decide which methods might work on the functional groups in your molecule, and then you can choose to study any aspect of the method as your group project.

For example, if bleach looks like a good method to try, your group might study the effect of bleach concentration, or you might try heating or cooling the bleach solution to see whether temperature has any effect on the neutralization. If you decide to try photodegrading your insecticide, you might try different irradiation times, or visible vs UV light, or short wave vs. long wave UV light. Activated carbon is good at absorbing many compounds, but you could try silica gel, or chicken feathers, or even soil. Alternatively, you could test one remediation method against another to see which one works best for your insecticide.

At the end of the discussion, we will write up a basic procedure for each group member to use in lab. These will be minor modifications of the procedures listed below. After each remediation method is used, you will assay the product to see whether it is less toxic, more toxic, or unchanged.

A. Basic bleach (oxidation and hydrolysis)

In a 125 ml conical flask are mixed 25 ml of 100 X solution, 0.5 g K_2CO_3 , and 5.0 ml of commercial bleach (e.g. Chlorox, 5.25% sodium hypochlorite). The reaction mixture is stirred magnetically for 20 minutes. The remaining bleach is neutralized by adding 0.25 grams of sodium metabisulfite and stirring for 5 more minutes.

B. Copper-catalyzed hydrolysis (hydrolysis)

In a 125 ml conical flask are mixed 25 ml of 100X solution and 0.25 g copper sulphate ($CuSO_4 \cdot 5H_2O$). This mixture is boiled for 30 minutes. Excess copper is removed by adding 3.0 g of ion-exchange resin, stirring for 5 minutes (the resin will turn light blue) and filtering off the resin.

C. UV treatment (photodegradation)

25 ml of 100 X solution is exposed to bright sunlight or a UV lamp for 40 minutes.

D. Activated carbon (adsorption)

25 ml of 100 X solution and 5 g of activated carbon are stirred at room temperature for 20 minutes, then the liquid is filtered.

VII. Laboratory supplies required for remediation lab

22 students

Item	Quantity
1. Disposable 1 ml transfer pipets, graduated	~ 500 (1 box)
2. 13 mm test tubes	240
3. Test tube racks (must hold at least 10 test tubes) or 500 ml beakers	22
4. 250 ml conical flasks (500 ml also OK)	110
5. 2 L conical flasks	7
6. 100 or 50 ml graduated cylinders	22
7. 10 ml graduated cylinders	7
8. Latex safety gloves (lg)	1 box
9. Acetylation vials	22
10. Labels (small)	1 roll
11. Parafilm and scissors	1 roll
12. Plastic weighing boats	36
13. Flea dips	7 bottles
14. Brine shrimp	1L batch
15. Instant Ocean	500 g
16. Yellow rubber gloves (2pr each sm, med, lg)	6 pr
17. Stir bars and magnetic stirrers/heaters	7
A. Per person: Chlorox bleach 5 ml, potassium bicarbonate 0.5 g, sodium metabisulfate 1 g, starch/iodine paper	
B. Per person: Copper sulphate pentahydrate 0.25 g, cation exchange resin in Na form (eg Bio-Rex 70) 3 g, gravity filtration setup.	
C. Per person: Petri dish, sunlight or UV lamp (a lamp for visualizing of TLC plates is fine)	
D. Per person: Activated carbon 5 g, gravity filtration setup.	

VIII. Lab report instructions

Each member of the group is responsible for writing a lab report for me (in my persona as your Cow Chemical Company supervisor). You can hand in your report in class on April 23, or get it to my office by 4:00 on Friday, April 24. (We do not have lab that day) The report should consist of the following sections:

1. Your lab sheet from the LD-50 lab on April 3.
2. The library research assignment for your insecticide product
3. A report on what you did in lab on April 17. This section will have a strict **3-page limit** and should consist of the following:
 - a) Brief description of the group's research plan and where your experiment fits into it.
 - b) Excruciatingly complete description of what you did in lab. The goal of this is to enable another person to do exactly the same thing that you did (and hopefully get the same results). This means you need to say what items of glassware or equipment you used for each step, describe the way the solutions looked, describe anything that went wrong, and of course, show your data.
 - c) Analyze your data as you did for the first lab to get LD-50 and LE-50 values.

The lab reports will be graded individually.

IX. Group poster instructions

There are many ways to convey scientific information; the **poster session** is quite popular for up-to-the-minute results. A poster is a lot like the display for a science fair project. It is a visual method of communication, so readability and appearance both matter.

Your group is jointly responsible for producing a poster describing your research project. On Tuesday April 28, each group will set up its poster, and you will have an opportunity to appreciate your classmates' creativity, scientific talent, and graphic design ability. In addition to your grade, fascinating prizes are at stake.

What to include in your poster:

- I. An introduction that a) sets the problem in context and b) describes the question that you tried to answer.
- II. Background material aimed at helping the non-specialist to understand the experimental approach. Many people have never heard of brine shrimp, LD-50 tests, specific methods of remediation, or the molecular structure of your insecticide--but they need to have some inkling of these things in order to understand the experiment you did.
- III. Experimental procedure and results. Describe what was done, and what the results were. It is appropriate to use graphs or tables to show results, but try to avoid having three nearly identical figures appear side by side.
- IV. Discussion of results. This is the section where your group must answer the questions that were raised in the introduction using the results of your experiment. If your experiments were inconclusive, that's fine. If you are left with unanswered questions, whether old or new, it is nice to include some ideas for what you would do in further research.

Your group will have a 3' X 5' poster board. 12 pages are the maximum that will fit in this space--but bear in mind that the poster format requires that you use enormous type faces. The absolute smallest font size you can get away with is **14 point (and 18 is much better)**.

Complicated molecular structures, graphs, and drawings should occupy 1/3 to 1 sheet of paper, and should also have large-sized labels and lettering. A good rule to go by is that everything on the poster should be legible from a distance of 4 feet away. There are many nice posters displayed in the halls of Stepan, Nieuwland, and Galvin. A "field trip" to see some of them will give you an idea of the format and types of layout that can produce an effective display of scientific results.

The group grade will include 25 points for presentation (readability, appearance) and 50 points for content (sections I-IV above).

X. Group poster grading sheet

_____/25 Presentation (Readability, appearance)

_____/50 Content

Introduction

Background

Experimental procedure

Results

Discussion of results

_____/75 Overall grade

XI. Literature Cited

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