A SIMPLIFIED ACUTE TOXICITY TESTING PROTOCOL With *CERIODAPHNIA DUBIA*

Prepared for:

Alameda Countywide Clean Water Program
Contra Costa Clean Water Program

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November 1997

1.0 INTRODUCTION

This protocol provides guidance to students and individuals interested in testing the toxicity of waters in their environment, using the laboratory test organism *Ceriodaphnia dubia*.

1.1 Background

Ceriodaphnia dubia is a small crustacean found in vernal pools and in freshwater ponds and lakes throughout the world. Female *Ceriodaphnia* can produce offspring without fertilization, a process known as parthenogenetic reproduction, when food supply is ample and the quality of the water is good. The organisms can sense when the water quality deteriorates, for example when a vernal pool is gradually drying out, and give birth to males. Fertilized females produce special eggs that can survive a long time without water and hatch when freshwater is introduced again.

Given the appropriate food and fresh water, female *Ceriodaphnia* can keep multiplying in a jar for a long period of time. Thus, they can be grown in **"culture"** at home or in a laboratory. *Ceriodaphnia* is very sensitive to pesticides, heavy metals, and other toxic substances used by humans and discharged into surface waters. These properties make *Ceriodaphnia* a good organism for testing the **toxicity** of freshwater. Natural waters can become poisonous to the organisms that live in those waters when pollutants enter the water in too high a concentration. Toxicity refers to the effect on aquatic organisms, rather than to the concentration of the pollutants.

In a typical toxicity test, *Ceriodaphnia* placed in **"test chambers"** full of sample water are periodically observed for a given length of time, for example 48 hours, and their survival (or death) is recorded. In addition, some *Ceriodaphnia* are placed in test chambers full of clean, healthy water to provide an experimental **"control"**. If the organisms in the control live and the organisms in a sample die, we know that they were initially healthy and something which is present in the sample (but not in the control) had caused their mortality. The water sample is considered "toxic". But if they die in the control as well, we know that something was wrong with the entire test (for example, the incubation temperature was too high) and the test is not valid.

The US Environmental Protection Agency (EPA) has developed a detailed protocol for toxicity testing, using *Ceriodaphnia* grown in culture to warn us that toxic substances are present in the waters around us. The test protocol is used by numerous laboratories across the nation to test effluents of wastewater treatment plants and urban runoff samples. To make sure that all the laboratories are using *Ceriodaphnia* cultures that have similar sensitivity to toxic substances, laboratories are required to run a **"reference toxicant"** test in parallel to sample testing.

Reference toxicant solutions are prepared at known concentrations from purified substances such as salts (e.g., potassium chloride) or heavy metals (e.g., copper sulfate). The response of *Ceriodaphnia* to these substances has been tested numerous times, and a characteristic range of concentrations that causes mortality has been established for each reference toxicant. If a reference toxicant test is run in parallel with sample testing (using the same batch of organisms from a single culture) and the organisms are responding in their characteristic way, the test is valid. But if the response to the reference toxicant is not within range, e.g., the organisms do not die when exposed to toxicant concentrations that normally kill them, this is a reason to invalidate the sample results because we are not sure that the organisms of this batch were sensitive enough to detect toxicity. Similarly, a test would not be valid if the organisms died when exposed to a reference toxicant concentration that does not normally kill them.

1.2 Scope, Limitations, and Organization

The purpose of this protocol is to provide guidance to students and individuals interested in testing the toxicity of waters in their environment. In preparation of this protocol, the stringent requirements of the EPA protocol (which can be met only in a fully equipped toxicity testing laboratory) have been modified for simple facilities that are feasible in homes and classrooms. However, the essential elements of the toxicity test have not been compromised. Results of tests run according to this Students' protocol will answer the question "Is it toxic?" in statements such as "it is lethal to *Ceriodaphnia* within 48 hours" or "it does not kill *Ceriodaphnia* within 48 hours". However, the results will not answer the question "how toxic is it?", i.e., will not provide information on the absolute intensity of toxicity.

The following section of this protocol (Section 2) lists some tips for short-term maintenance of cultures for the duration of a project period (4-6 weeks). Section 3 provides advice for sample collection and storage, and Section 4 explains how to use the data sheets. Section 5 gives instructions for toxicity test setup, assuming that all materials and equipment listed below are available. Section 6 describes activities related to daily observations, record keeping, and using the data sheets. Section 7 provides reporting formats and statements. All data sheets and forms are provided at the end of this protocol, Section 8.

Appendix A contains a list of equipment and materials (quantities and sources) required for each test or project period, including a list of persons to contact in San Francisco Bay Area commercial laboratories for *Ceriodaphnia* cultures and food supply. Appendix B summarizes the quality assurance/quality control elements contained in this protocol and discusses the data quality objectives for some parameters. Appendix C provides instructions for data entry into an Excel spreadsheet template that creates a survival curve and a summary table that can be copied directly into the regional toxicity database.

1.3 Materials and equipment.

This list includes the materials and equipment needed to maintain cultures and conduct toxicity tests. The sources and quantities of these items are provided in Appendix A. It must be noted that the concentrations given for the reference toxicant solutions are preliminary and require verification by more tests.

Ceriodaphnia culture starter (60 organisms or more)

YCT mixture
Selenastrum concentrate
Syringe without needle, 5 ml
Clear, wide mouth glass jars without lids for *Ceriodaphnia* cultures, 800-1000 ml
Clear, wide mouth glass jars with lined lids, 250 or 500 ml, for samples
"Alconox" detergent for jar cleaning
Arrowhead Spring Water
Evian mineral water
Distilled/purified water, supermarket grade
one 9 oz clear plastic cup, labeled "Wasser"
2 Plastic 9 oz cups labeled "RT1" and "RT2"
one 60 ml plastic syringe without needle, or 100 ml graduated cylinder, for Wasser
one 5 ml plastic syringe without needle, to dispense KCl solution
Reference toxicant (Reftox) stock solution (KCl 10 g/l)
"Cerio cups" (Solo 1 oz)
Disposable 9 oz clear plastic cups
Permanent marking pen
Hand lens (or better still, microscope) to observe dead and living *Ceriodaphnia*
Small bulb thermometer
"Cerio board" (bottoms of egg cartons, or a Styrofoam board with holes for cerio cups)
Refrigerator

plastic pipette cut at the end
one 100 micron sieve
one 400 micron sieve
one small flat cup (2 or 3 oz Solo plastic cup without lid)
one conductivity meter
one pH meter or a pack of non-bleeding pH strips
Minimum-maximum thermometer

Data sheets and forms (All forms are provided at the end of this protocol, Section 8).

2.0 SHORT TERM MAINTENANCE OF *CERIODAPHNIA* CULTURES

Professional toxicity testing laboratories will provide a starter culture and food mixtures adequate for about one month of culture maintenance. Keep the food mixtures refrigerated all the time. Use the culture log sheet "*CERIODAPHNIA* CULTURE LOG" to keep daily records of the culture.

Containers: At school or at home, *Ceriodaphnia* cultures can be maintained in glass jars of about 1 liter (1 quart), subsequently referred to as "culture jars", without lid. The jars can be placed on a shelf or window sill but away from direct sunlight in a location that is not accessible to pets or small children and not exposed to household chemicals. Culture jars that have been used before must be cleaned carefully with dishwashing soap followed by a thorough rinse in tap water.

Water: The culture can be maintained in the control solution nicknamed "Wasser water" (because it is a mixture of two waters, and wasser means water in another language) or just **"Wasser".** Wasser is a moderately hard water prepared by mixing Arrowhead spring water (80% volume/volume, or v/v) with Evian mineral water (20% v/v). In other words, to prepare one liter you will mix 800 ml of Arrowhead with 200 ml of Evian and shake well (i.e., to make just over 1 quart, mix 4 cups of Arrowhead water with 1 cup of Evian water). Some change in pH (how acidic or alkaline the water is) will be observed during the first 24 hours, but this does not seem to affect *Ceriodaphnia* (both fresh and stored mixtures are OK). Wasser can be stored at room temperature, already mixed, for more than six months.

Feeding: Each day each culture jar should get 4 ml of *Selenastrum* concentrate (the green algae mixture) and 4 ml of YCT (the brown mixture, containing goodies such as yeast, crushed alfalfa leaves, and digested trout chow). Use the 5 ml syringe without needle to dispense food solutions (first algae and then YCT) and rinse it immediately in tap water. If the culture is not visited during the weekend, add 8 ml of each food mixture to each jar on Friday. Do not overfeed! *Ceriodaphnia* can go a long time without food but may die of oxygen depletion if too much food is added. You will have visual cues: if the turbidity ("cloudiness") of the water in the jar is high (e.g., you cannot clearly see the outline of a dime on the other side of the jar) it means you added too much food. If you see tiny organisms (= neonates, or babies) in the culture you know that the culture is getting enough food and is reproducing well. When you are planning to set up a toxicity test, feed the culture 1-2 hours before setup.

Subculturing: The culture needs to be diluted and "renewed" once a week. To do that, pour about 250 ml culture, containing 40 or more organisms, into a fresh empty culture jar (this will be about one quarter of the jar). If all the organisms are at the bottom, mix the culture gently before pouring. Mark the date and Jar # on the fresh jar, and then pour Wasser into the jar up to three quarters of the volume (Culture liquid should fill about three-quarters of the volume of the jar to allow for some air space). Then, add 250 ml to the older culture jar and keep on the same shelf. It is prudent to keep at least two culture jars with *Ceriodaphnia* at all times, and if a big test is planned, to keep more than two.

Water Quality Monitoring: Because we want to assure healthy, physiologically comfortable conditions, monitoring of temperature, pH, and conductivity is recommended. Make sure the tip of the pH meter has been kept damp, or soak it in tap water for 20 minutes before calibrating according to manufacturer's instructions. Always calibrate the pH meter and rinse it well before measuring culture pH. When taking measurements, wait several minutes for the reading to stabilize. If you are using pH strips, transfer a small volume of culture water to a small cup (e.g., cerio cup), use only the non-bleeding type (e.g., colorpHast), and wait several minutes before you take your reading. waiting is very important when you measure the pH of low-buffer solutions, either with the pocket meter electrode or with the pH strip.

Measure and record pH and conductivity once a week, just before subculturing, and if the pH exceeds 8.6 incubate your cultures in a less illuminated (lighted) area. Electrical conductivity (EC), a measure of the amounts of salts in the water, is expected to remain in the range of 200-500 microsiemens (μS). Place a minimum-maximum thermometer near the culture jar and measure air temperatures daily to characterize your incubation area, recording the minimum, current, and maximum temperature (min/curr/max). Temperatures of 18-25 degrees Celsius are reasonable. *Ceriodaphnia* are tough and will survive short exposures to 5 or to 30 degrees Celsius, but extreme temperature will stress them and may make them more sensitive to toxicants.

3.0 SAMPLE COLLECTION

Although this protocol focuses on testing the toxicity of urban runoff during storm events, it can be used for any other type of sample, e.g., dry weather creek samples, discharges from outfalls, etc.

Sample containers: Samples should be collected in glass jars with caps that have inert lining (not paper etc.) or could accommodate an empty plastic bag as cap lining without leaking. The minimum volume required for one test is about 100 ml (about 3.5 fluid ounce), but using jars of 250 or 500 ml is recommended. Larger jars will allow repeating the test or performing other tests and analyses if necessary. Jars should be cleaned with “Alconox” (a special dishwashing detergent that does not leave residues), followed by a thorough rinse in tap water.

Stormwater sample collection: Urban runoff samples may be collected in street gutters, creeks, or roof downspouts (particularly from tar-and-gravel roofs). Make sure it is safe to access your sampling location during the storm**. *Note: Do not enter flood control channels without authorization. Do not cross private property without permission. Do not take samples at a creek until you have reviewed the safety sheet.*** Samples may be collected at different times during a storm event, but if only one sample can be collected the best time is after the initial wave of dirty water has gone by and before the flow begins to subside. Make your own estimate but keep records of rain information, time and flow if you can. It may also be interesting to test samples collected from outfalls that discharge water into the creek during dry weather. Before you collect a sample, each sample container must be labeled with a unique Sample ID (e.g., your initials plus a serial number), sampling date, time, and location. Immediately after the sample was collected, fill out a “FIELD DATA SHEET FOR STORM RUNOFF TOXICITY STUDY” for that sample. The sheet will serve as a “chain of custody” form and should be attached to the other data sheets (see below). All forms are provided at the end of this protocol.

Sample storage: Store samples in a refrigerator, and start the test ASAP within 36 hours (if you are sampling a constant source and are able to take samples anytime) or within 72 hours (if samples are collected during a rain event).

4.0 RECORD KEEPING

Tidy record keeping is essential if the data are to be used by anybody other than you. This part of the protocol contains two data sheets:

- DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST Page 1: Control and Reftox
- DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST Page 2: Samples

Use the 2 data sheets for each test, one for control and reference toxicant (**"Reftox"**) data (page 1) and the other for sample data (page 2). If you test more than three samples, use another page 2 sheet and mark it as Page 3.

Before you start the test, record Test ID on both pages.(e.g., SLH-E2 for San Leandro High, storm event 2). On Page 1, record all the test information in the box at the top: culture, reftox, project and team members, city/county/state, etc. On Page 2, record all the sample information in the box at the top : Sample ID, sampling date and time, location, time after rain started, and sample description (e.g., turbid, yellow, oily sheen). Record room temperature and date (not the time) under the "To" column on the center box (Schedule) on both pages. Record the Sample ID in the column left of the "survival" center box for a block of 4 rows. The number "5" has already been added under the "To" column. Subsequently, use the data sheets to record all test information as instructed below.

5.0 SETTING UP A TOXICITY TEST

5.1 Preparation of test solutions

5.1.1 Decide what is the number of "treatments" (e.g. 3 samples, 1 control, 2 reftox) you can handle. Label one new 9 oz disposable clear plastic cup for each sample, using the sample ID. Label three cups for Wasser, RT1 and RT2 treatments. These may be recycled (from test to test, if you rinse them with tap water) so they may be already labeled.

5.1.2 Wet the 100 microns sieve with deionized water (DI) or distilled water. Filter sample water through the 100 microns sieve into labeled 9 oz disposable clear plastic cup, about 100 ml (1/2 full). Wash sieve immediately and between samples, using tap water followed by DI or distilled water.

5.1.3 Pour about 100 ml of Wasser (control water) into the Wasser cup. Dispense exactly 98 ml Wasser (plus or minus 2 ml is OK) into the first reftox cup, the 9 oz cup labeled RT1, using the 60 ml syringe or the graduated cylinder. Then add 1.5 ml of the 10 g/l KCl stock solution (plus minus 0.1 ml is OK) into that cup, using the 5 ml syringe, and mix well; you now have 150 mg/l of KCl in your RT1 9 oz cup, ready for the test. Use a similar dilution process for RT2: dispense 95 ml of Wasser and 5 ml of KCl stock solution to yield a final concentration of 500 mg/l KCl.

5.1.4 Set all your test solutions aside until water reaches room temperature.

5.2 Preparation of adult *Ceriodaphnia* for the test

5.2.1 Prepare a pile of empty, new "**cerio cups**" (Solo 1 oz). You will need four replicates for each treatment, so if you plan to have 6 treatments (3 samples, 1 control, and 2 reftox) you need 24 cerio cups (6 treatments X 4 replicates = 24). Using a permanent marking pen and writing on the outside of the cups close to the top, label each cup with ID of treatment and a replicate letter from A to D (e.g., RT1-C). Place the labeled cerio cups randomly on the table.

5.2.2. Place the 400 micron sieve in the small flat cup (Solo 2 oz) and add a small volume of Wasser (just to make sure that the net is wet. Then, while holding the sieve inside the flat cup and above a deep tray or a wide mouth jar, pour *Ceriodaphnia* culture through the sieve. The liquid will fill the flat cup and flow into the tray or the wide-mouth jar. *Always keep the sieve in liquid in the flat cup.* ***Never strand the animals on a drained sieve!*** *This may cause mortality later.* You will notice that the smaller *Ceriodaphnia* pass through the sieve. After passing most of the culture volume through the sieve (taking care not to suspend the material at the bottom of the culture jar), pour a small amount of Wasser through the sieve to wash out the animals that are smaller than 400 microns. Save the culture liquid and Wasser that had drained into the tray or the wide-mouth jar, it contains your future culture. You will need 120 adults for each test of 6 treatments. Use the second culture jar to collect more adults if needed.

5.2.3 Pipette 5 adult *Ceriodaphnia* from the sieve into each labeled cerio cup (taken at random from the table) and leave them in a small volume of water. Do not agitate; this may move the drop and strand animals on the dry plastic surface of the cerio cup. Place the cups with animals in groups according to treatment (e.g., all the cups, replicates A,B,C, and D of the sample RT2 in one group) at different corners of the table.

5.3 Adding sample, control and reftox solutions to cerio cups

5.3.1 For the first treatment, e.g., RT1, mix the content of this treatment's solution in the 9 oz disposable clear plastic cup (should be at room temperature by now), and pour about 15 ml (about 2/3 of cup's height) into each of the four cerio cups with animals in the group you have labeled and prepared for this treatment. You should have about 1 inch of solution left in the 9 oz cup. Save it for water quality testing (see below, section 5.4). Place the 4 cerio cups of each treatment randomly on the cerio board.

5.3.2 Proceed in the same manner for each of the treatments, and place filled cerio cups randomly on cerio board.

5.3.3 When you have completed adding solutions to all treatments, record the time under "To" in the data sheets. Observe all the cups in the cerio board, and if you see *Ceriodaphnia* that are floating on the surface, use one drop of treatment solution dropped from a (clean) pipette directly on the floating animal to bring it back into the water column. Wash the pipette thoroughly with DI if you need to sink floaters in other treatments.

5.3.4 (optional) Place three additional cerio cups with tap water, labeled "temperature" in bold letters (different color sharpie?) at different locations on the cerio board. These cups will be used to measure temperature during each observation.

5.3.5 Cover the full cerio board with a transparent, rigid cover (e.g., Plexiglas) and place it on a shelf or a table, never in direct sunlight, for “incubation” at room temperature. Place the minimum maximum thermometer adjacent to the cerio board. You can also incubate *Ceriodaphnia* in the dark, but avoid drawers and unventilated cupboards. Record the incubation setup on the back of your data sheet.

5.4 Measurements of water quality parameters (initial values)

The pH and electrical conductivity (**EC**) are measured at the beginning and at the end of the test, using the meters in the same way as described in Section 2 above (see also the Water Quality monitoring protocol developed for citizen volunteers). After setting up the test, measure the pH and electrical conductivity in the remaining solutions in the 9 oz disposable clear plastic cups. Record under “initial values” on bottom of the two data sheets. If the conductivity meter shows only the digit “1” at the left end of the window, this means the value is above 1990 microsiemens (µS), and the sample needs to be diluted in distilled water for measurement of conductivity. To do this, take a new cerio cup, fill it to the top with sample solution, and pour it into a new 9 oz cup. Then pour distilled water into the cerio cup (to the top) and pour that into the same 9 oz cup. Mix well, measure the EC, multiply this value by 2 and record in the data sheet. Make a note that the sample was diluted to 50% for EC measurement.

6.0 DAILY OBSERVATIONS

6.1 Observe all the cerio cups twice a day, preferably at 12 hours intervals. Try to make observations as early as you can in the morning and as late as you can at the end of the day. Make an effort to make the last observation as close as possible to 48 hours from test setup (To). If you cannot perform two observations each day, have one at 24 hours and one at 48 hours.

6.2 For each observation, record the date and time in the appropriate row (under the T1, T2, T3, or the T4 columns) on Page 1 and Page 2. Record your name or initials in the “observer” row on Page 1 and Page 2. Record air temperature: minimum, current (what the thermometer reads at time of observation), and maximum, on Page 1. Record the water temperature (average of three measurements with a small bulb thermometer in “temperature” cups) for that time on page 2. For air temperature, you can record the min-max values in the same cell on either side of the current temperature (e.g., 19/21/22). Reset the minimum-maximum thermometer.

6.3 Calculate how many hours elapsed since the test was set up and record the number of hours in the “exposure duration” row.

6.4 During observation, determine the number of surviving *Ceriodaphnia* in each replicate of each treatment, as well as the number of dead ones, and record it in the appropriate cell in the “survival” center box. A *Ceriodaphnia* is considered dead if it stays on the bottom of the cup and does not move after the cup is tapped or swirled lightly. Record what you see, even if you are not completely sure. *Do not erase any records if you find more animals alive in the next observation. This kind of mistake* *happens to professional, experienced toxicologists all the time and it's OK; just keep recording what you see*. You may see tiny animals in the cup, young *Ceriodaphnia* that were born during the test. Do not include those in the number of dead and surviving animals; concentrate on the five large adults that were initially placed in the cup, and it is also helpful to record the number of dead adults you see. *Note: Crustaceans have an external skeleton that undergoes molting every once in a while. The molts are normally transparent, but some Ceriodaphnia may have aborted broods inside the molts and this will look like a dead one under the hand lens. Use a microscope if you have access to one.* Either way, keep counting the live, large adults.

6.5 After the last observation, measure pH and conductivity (EC) in one replicate of each treatment and record under "final values". Avoid placing measuring instruments or pH strips in cerio cups that are still in incubation (i.e., during the test); this may harm the organisms and produce false results. When you terminate the test, you may keep the surviving organisms in a separate jar that will never be used for toxicity testing (not even control survivors), or send all the organisms used in the test to "*Ceriodaphnia* heaven" by adding table salt.

7.0 REPORTING OF TEST RESULTS

This section provides instructions for manual calculation and plotting of test results. The user is also referred to Appendix C of this protocol, which provides instructions for data entry into an Excel spreadsheet template that creates a survival curve and a summary table that can be copied directly into the regional toxicity database.

7.1 At the end of the test, calculate the percentage of surviving *Ceriodaphnia* in each treatment (sum of four replicates) for each observation T1 through T4, and record it in the "percent survival" row under this treatment's block. Plot the percent survival as a function of exposure duration, for the control and for each sample, using different symbols.

7.2 To validate the test, compare the results of the control and reftox treatments to the test validation criteria of performance. These criteria are:

- at least 80% survival in the control;
- at least 80% survival in reftox 1 (150 mg/l potassium chloride),
- 50% survival or less in reftox 2 (500 mg/l potassium chloride).

7.3 To examine whether the observed effects may have been due to unfavorable physiological conditions associated with basic water quality parameters, compare the water quality measurements to the acceptable ranges:

- pH in the range of 6.5-8.8,
- conductivity 40-3000 microsiemens (μS),
- temperature 10-28 degrees Celsius.

7.4 Prepare summary statements. Examples:

"The toxicity test was valid in terms of control and reftox performance"

“Initial and final pH and conductivity were within acceptable range”
“Sample ….(name ID) collected at….(name location) was lethal to …. % of *Ceriodaphnia* within 48 hours”

8.0 DATA SHEETS AND FORMS

CERIODAPHNIA CULTURE LOG (FormTTS30)
FIELD DATA SHEET FOR TOXICITY STUDY SAMPLING (Form TTS35)
TIPS ON COLLECTING STORMWATER SAMPLES (Form TTS36)
DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST Page 1: Control and Reftox
DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST Page 2: Samples
Ceriodaphnia Survival Curves

CERIODAPHNIA CULTURE LOG

School/Program_____________________________ Page ________ of ______

Daily Feeding

Date	Time	Operator	Jar #	YCT added (ml)	Algae added (ml)	Air temperature (min/curr/max)	comments

Subculture Records

Date	Time	Operator	Jar #	Final pH	Final EC

Separation of adults for toxicity tests

Date	Time	Operator	Jar #	Size cutoff (sieve pores)

Form TTS35

FIELD DATA SHEET FOR TOXICITY STUDY SAMPLING

Use this data-sheet during your sampling activity, either for sampling during dry weather or for sampling during a storm event. Fill one sheet for each sample you collect, using Section A. If you have information for Section B, please provide it. Add any other information that may be relevant. Attach these data sheets (in a Ziplock bag) to your samples.

Section A

Sample ID (your initials plus serial #)	Collection Date	Collection Time	Hours since rain started	Collector's Name (print) and phone #	Sample source or type (rooftop, gutter, creek, outfall, etc.)
Sampling location: city, (state), street address, orientation (north, southeast, etc.) in relation to street, or creek					

Section B

Sample water description: turbidity, color, smell
Water Quality: temperature, conductivity, pH
Water level in relation to landmark, is level rising or falling when sampling
Estimated Flow (cfs, gallons/minute)

Comments:

Sample received (Date & time)__________________________by__________________________________

TIPS ON COLLECTING STORMWATER SAMPLES (FORM TTS36)

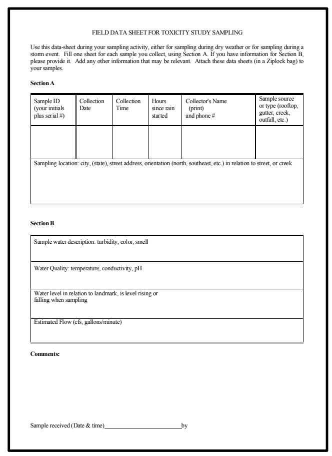
Samples should be collected in clean glass jars with caps that have an inert lining (plastic, not paper or foil). If you only have jars with paper or foil in the caps, see if an empty plastic bag can be screwed under the cap lining without causing leaks. Use 250 ml (8 ounce) jars and fill them completely so there will be enough sample for more analyses if needed.

Urban runoff samples may be collected in street gutters, creeks, or roof downspouts (particularly from tar-and-gravel roofs). Make sure it is safe to get to your sampling location during the storm**.** Samples may be collected at different times during a storm event, but the best time is after the initial wave of dirty water has gone by and before the flow begins to subside.

Before you collect a sample, label the jar with:

- a unique Sample ID (such as your initials plus a serial number)
- sampling date
- sampling time

Rinse the jar two times with sample water and then fill the jar and cap it. If the water is too shallow to dip the jar all the way in, use the lid to scoop water into the jar.



FIELD DATA SHEET FOR TOXICITY STUDY SAMPLING

Use this data-sheet during your sampling activity, either for sampling during dry weather or for sampling during a storm event. Fill one sheet for each sample you collect, using Section A. If you have information for Section B, please provide it. Add any other information that may be relevant. Attach these data sheets (in a Ziplock bag) to your samples.

Section A

Sample ID (your initials plus serial #)	Collection Date	Collection Time	Hours since rain started	Collector's Name (print) and phone #	Sample source or type (rooftop, gutter, creek, outfall, etc.)
Sampling location: city, (state), street address, orientation (north, southeast, etc.) in relation to street, or creek					

Section B

Sample water description: turbidity, color, smell
Water Quality: temperature, conductivity, pH
Water level in relation to landmark, is level rising or falling when sampling
Estimated Flow (cfs, gallons/minute)

Comments:

Sample received (Date & time)________________by

Immediately after you collect the sample, fill out a Form TTS35, the "FIELD DATA SHEET FOR TOXICITY STUDY SAMPLING" for that sample.

Be sure to fill in the exact location, providing enough information on the data sheet that another person could go back to the same spot by following what you have written. This is extremely important!

Keep the data sheet with the sample at all times; it is also a Chain-of-Custody sheet (a record of who had the sample between the time it was collected and the time it was tested).

Store the samples in a refrigerator or cooler, and bring them to your teacher along with the field data sheet as soon as possible.

Remember, **SAFETY COMES FIRST**!

- *Do not enter flood control channels without authorization.*
- *Do not cross private property without permission.*
- *Do not take samples at a creek until you have reviewed the safety sheet***.**

DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST   Page 1: Control and Reftox

Test ID ____________

Top box: **Test Information**

Culture Source: ___________

School: ____________________________

Date culture started: _____________

Project: ____________________________

Reftox Source: ________________

Team members: ____________________

Center box: **Schedule**

	To	T1	T2	T3	T4
Date					
Time					
Temp. (air)					
Observer					

Exposure duration

Center box: **Survival**

Control

Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

Reftox 1

Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

Reftox 2

Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

Bottom box: **Water quality**

Treatment	Initial pH	Initial EC	Final pH	Final EC
Control				
Reftox 1				
Reftox 2				

DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST   Page 2: Samples

Test ID ____________

Top box: Sample Information

Sampling Location	Sample ID	Date Collected	Time Collected	Time rain started	Sample description

Center box: Schedule

	To	T1	T2	T3	T4
Date					
Time					
Temp. (H2O)					
Observer					

Exposure duration

Center box: Survival

Sample ID

(Sample 1)

Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

(Sample 2)

Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

(Sample 3)

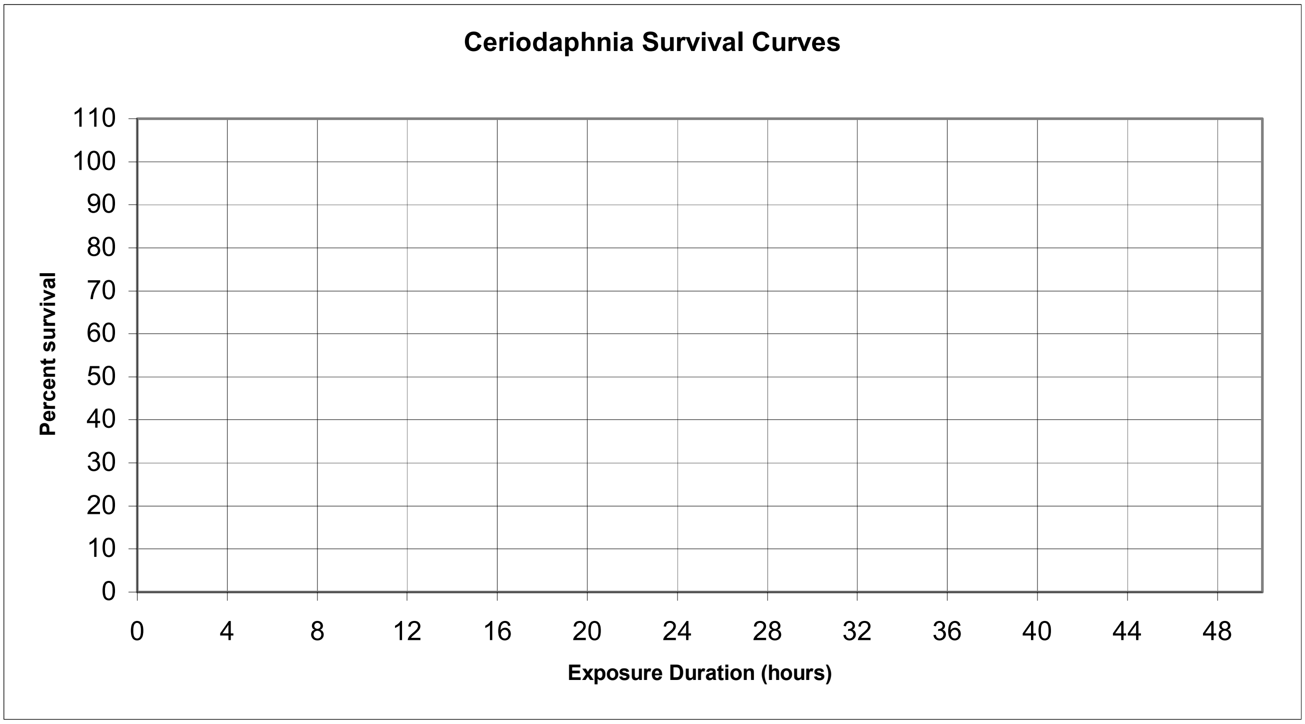
Replicate A	5				
Replicate B	5				
Replicate C	5				
Replicate D	5				

Percent survival

Bottom box: Water quality

Sample ID	Initial pH	Initial EC	Final pH	Final EC

Test ID Test date Program



Legend: x - control , - Sample - Sample - Sample

Result Statement:

Comments:

APPENDIX A: EQUIPMENT INVENTORY

The following list includes all materials, solutions and equipment needed to run *Ceriodaphnia* toxicity tests using the SIMPLIFIED PROTOCOL. The list is divided by potential sources of these items.

1.0 Toxicity Testing Kit (to be supplied to teachers etc. by a government agency)

<u>Disposable equipment and materials:</u>

one syringe without needle, 5 ml, for feeding
one syringe without needle, 60 ml
one syringe without needle, 5 ml, for KCl
plastic pipette with built in bulb, 5 ml, cut at the end
cerio cups, 24 per test of 6 treatments plus 3 for temperature measurements (1 oz Solo plastic cups without lids)
stock solution 10 g/l potassium chloride (KCl) in distilled water
"Alconox" detergent for jar cleaning, a few grams for the entire project

<u>Equipment on loan</u>:

one 100 micron sieve
one 400 micron sieve
one small flat cup (2 or 3 oz Solo plastic cup without lid)
one conductivity meter
one pH meter or a pack of non-bleeding pH strips
Minimum-maximum thermometer

2.0 **Test organisms and food (To be supplied by local toxicity laboratories)**

Ceriodaphnia culture starter (40 organisms or more)
YCT mixture, 120 ml per month
Selenastrum concentrate, 120 ml per month

Participating toxicity laboratories (1998):

Pacific Eco-Risk, Martinez (Scott Ogle, (510) 313-8080)
Block Environmental Services, Pleasant Hill (Ron Block, (510) 682-7200)
MEC, Tiburon (Paul Krause, (415) 435-1847)
ToxScan, Watsonville (Dave Lewis, (408) 724-4522)

3.0 **Local grocery (to be purchased by user)**

Arrowhead Spring Water, 1 gallon per month
Evian mineral water, 1 liter per month
Distilled/purified water, supermarket grade

Disposable 9 oz clear plastic cups, 3 or more per test

4.0 Classroom and home (Attic/garage/kitchen) items

Clear, wide mouth glass jars without lids for *Ceriodaphnia* cultures, 800-1000 ml

Clear, wide mouth glass jars with lids lined with inert material (or an empty sandwich bag), 250 or 500 ml, for samples

Permanent marking pen

Cerio board (bottoms of egg cartons, or a Styrofoam board with holes for cerio cups)

Flat flashlight under wire shelf or frame, and opaque white surface on top, for a Light Table (if needed)

Hand lens (or a microscope) to observe dead and living *Ceriodaphnia*

Small bulb thermometer

5.0 Other suppliers (1998)

If your school can spend $25-35 a month for *Ceriodaphnia* food, you may include supplier-information in your protocol. A company called Aquatic Biosystems Inc. (1-800-331-5916), located in Colorado, will ship *Ceriodaphnia* food by UPS overnight service (COD possible). In 1994 they charged $15 for 0.5 liter of *Selenastrum* food suspension and $10 for 0.5 liter of frozen YCT mixture; these quantities should suffice for 3-4 weeks.

The 400 micron net is commercially available under the trademark Nitex, which is a nylon netting. It may be purchased, for example, under Catalog # E-NT-NTX 400 from Argent Chemical Laboratories, 8702 157th Avenue North East, Redmond, WA 98052 (Telephone number 800-426-6258). Similarly, the 100 micron net is sold under catalog # E-NT-NTX-100. Pieces of one square meter are the minimum size sold, and it costs approximately $40.

APPENDIX B: QUALITY ASSURANCE GUIDELINES

This section identifies how accurate, precise, complete, comparable, sensitive, well documented, valid, and representative our toxicity testing and measurements will be, and what we can do to make them even better. In some cases we do not have enough information to provide a good evaluation (e.g., for representativeness or accuracy), due to constraints of our sampling design and to the nature of toxicity tests. However, if we are aware of these limitations, uncertainties, and sources of error, we can qualify the results so that any potential user will know how reliable our data are. Quality assurance/quality control (QA/QC) plans include several "elements" that are formally applied to assure and control the quality of data. The following guidelines discuss the applicability and utility of these QA/QC elements to our protocol.

B-1. GENERAL QA/QC CONSIDERATIONS

Record keeping / Chain of custody / Completeness

Use the data sheets provided throughout the protocol, as you work, and be sure to include all the information that the protocol is asking for. These sheets are also your reporting format, so they must be legible.

- Form TTS30*:* *CERIODAPHNIA* CULTURE LOG - to be filled daily by the culture maintenance crew
- Form TTS35: FIELD DATA SHEET FOR TOXICITY STUDY SAMPLING - to be filled by each sampler, for each sample, at the time of sampling. These forms are also your "chain of custody" records.
- DATA SHEET FOR *CERIODAPHNIA* TOXICITY TEST Page 1: Control and Reftox, and Page 2: Samples - to be filled when the test is set up and on every observation during and after the test.

It is assumed that every procedure of the test is performed according to the protocol, so there is no need to record these details again. However, if you add something or do something different, keep clear records of any additions to or deviations from the protocol.

Holding time : Generally, samples for toxicity testing should be refrigerated and tested as soon as possible or within 36 hours of collection from a constant discharge. However, if the samples were collected during a rain event or a temporary dry weather discharge, they should be tested within 72 hours.

Lack of Contamination: Sampling containers and laboratory utensils need to be carefully cleaned to assure that no toxicity, apart from the toxic substances potentially present in the sample (for which we are testing the sample), is inadvertently introduced.

Representativeness: This element is about how well the sample we have collected represents the environment that we have sampled, both in the temporal sense (e.g., what flows in the creek or street gutter during the entire storm event) and in the spatial sense (e.g., what flows in the same gutter at the same time 100 yards from where we are). The sampling suggestions provided in this protocol allow for a high degree of uncertainty about representativeness. However, if several samples are collected from a given environment, and pooled together, this can increase the representativeness.

B-2. QA/QC ELEMENTS SPECIFIC TO TOXICITY TESTING

Beyond the quality assurance/quality control (QA/QC) elements of sample collection, custody, and handling, QA plans for toxicity testing are designed to show that test procedures were adhered to, including water quality measurements, and that clear records are kept of any deviations from the protocol. These plans have procedures and criteria to show that all the test organisms were healthy and properly fed, that the control organisms survived and reproduced adequately, and that the organisms exposed to reference toxicants were not too sensitive or too resistant.

Test Validation Criteria:

1. Control survival should be at least 80%.

2. Reference toxicant tests are used to establish a laboratory's ability to obtain precise results, and also to establish an acceptable range of sensitivities of test organisms. This range is established for each combination of reference toxicant and test organism. For this protocol, potassium chloride (KCl) has been selected as a reference toxicant. The salt is used at two concentrations, reftox 1 (a concentration that is not expected to kill Ceriodaphnia more than 95% of the time) and reftox 2, a concentration that is expected to kill the organisms more than 95% of the time. The test validation criteria are defined as: at least 80% survival in reftox 1 (150 mg/l potassium chloride), and 50% survival or less in reftox 2 (500 mg/l potassium chloride). As of June 1998, the San Francisco Bay Area test results with these concentrations using adult Ceriodaphnia at room temperature has not been compiled to generate relevant statistics and confidence intervals.

Water Quality

Commercial laboratories testing environmental samples with *Ceriodaphnia* also has to show that the test organisms had enough oxygen, that the correct test temperature was maintained, that the pH values were not extreme, and that the organisms were not subject to osmotic stress. Water chemistry parameters are monitored daily during the test to ensure that the animals are exposed to environmental conditions which will not cause a "toxic effect" by themselves. The values should fall within the ranges known as "safe" for the organisms, and the laboratory is instructed to modify some parameters of the sample before exposure to prevent extreme ("out of range") conditions if necessary.

However, this simplified protocol for science students does not recommend modification of a sample before it is used for the test, and calls for measurements of pH and electrical conductivity (EC) at test initiation and termination only. In fact, this protocol calls for measurements of pH and EC so we can tell if mortality could be "explained" by environmental conditions that were not "safe", rather than change the conditions. The "safe", or "physiologically comfortable" conditions are: pH in the range of 6.5-8.8, and conductivity of 40-3000 microsiemens (µS). Temperatures in the range of 10-28 degrees Celsius (perhaps even lower temperatures) can be tolerated, but the test is conducted at room temperature rather than at the temperature at which the sample was collected. However, remember that any measurement outside these ranges does not invalidate the test.

Precision and Accuracy

The precision of a toxicity test is an expression of the degree of reproducibility of results, and it can be determined by evaluating the variability among laboratory replicates and by analyzing duplicate samples. Although this element has not been formally incorporated into the present protocol, variability among the four replicates can be evaluated, and duplicate samples may be analyzed from time to time.

Accuracy is the nearness of a measurement to its true value. In a biological toxicity test, accuracy is enhanced by using several replicate chambers for each sample. However, the "true value" of toxicity cannot be determined. This is because toxicity is a relative rather than an absolute concept, since only organisms can "measure" toxicity, and there is no true or absolute reference organism. Toxicity test results (e.g., percent survival) can be compared to each other, but their deviation from a true value cannot be determined. This is different from chemical quantification, in which standard analyte solutions or buffers are used to establish the true concentration or to calibrate instruments (see below).

Consistency / Comparability

Many times it is difficult to determine if a test organism is really dead, and different observers may have different "signs". The protocol provides a criterion to help make a decision (animal on bottom and does not move even after gentle tapping or swirling of the cup); this little "test" should be done when there is doubt. Consistent use of the same criteria, procedures, and data sheets by all programs will ensure that "everybody is on the same page", and will allow comparisons of the data. There is an inherent source of uncertainty about various sensitivities of test organisms from different laboratories. However, the use of reference toxicants provide a means of reducing uncertainties associated with organism sensitivity.

B-3. WATER QUALITY MEASUREMENTS

The precision of temperature or pH measurements can be formally evaluated by recording measurements of the same containers by different team members, measurements of different replicates of the same sample at the end of the test, etc. Duplicate samples are also useful, to account for the influence of sample jars. The degree of reproducibility of data is often expressed as "Relative Percent Difference" (RPD) which is the difference between the two readings, divided by the average of the two, and multiplied by one hundred. For example, if the conductivity readings of two duplicate samples were 180 and 220 microsiemens (µS), the RPD is (220-180)/200X100=20%.

To assure accuracy, instrument calibration for pH is recommended before each day-use, and for conductivity one time during the project. Thermometer readings should be compared to the best mercury thermometer available. In all measurements, the operator should be familiar with the time needed for equilibration or stabilization of the readings and wait until stabilization before recording the value.

The following table specifies the Data Quality Objectives recommended for Water Quality measurements:

Parameter	**Method/range**	**Units**	**Detection Limit**	**Sensitivity**	**Precision (RPD)**	**Accuracy**
Temperature	Thermometer (0 - 50^{o}C)	^{o}C	NA	1.0^{o}C	20%	± 1.0
pH	pH meter (3-12)	pH units	3	0.1 unit	10 %	± 0.3
	pH strip (non-bleed, 5-12)	pH units	5	0.5 unit	NA	± 0.5
Conductivity	Conductivity meter (10-1990)	umhos/cm	10	10 umhos/cm	20%	± 20

NA - not applicable
RPD - Relative Percent Difference - is the difference between the two readings, divided by the average of the two, and multiplied by one hundred.

ATTACHMENT 1: USING THIS PROTOCOL WITH *DAPHNIA MAGNA*

This protocol can be used for acute toxicity tests with *Daphnia magna*, but the following points need to be considered:

1. Culture jars are larger and culture density is lower for *D. magna*.

2. *D. magna* organisms may need a longer and more gradual acclimation period when transferred from the donating laboratory culture medium to Wasser.

3. *D. magna* test organisms are larger, so juveniles are used instead of reproducing adults. This eliminates the confusion arising from neonates appearing in test chambers during the test. Juvenile (3-6 days old) *D. magna* are separated from the culture in two steps: first, the entire culture is passed through a 1000 micron sieve and the filtrate collected in a clean container, while the adults (>1000 microns) are retained for a new culture; second, the filtrate is passed through a 500 micron sieve to collect the juveniles that will be used for the test.

4. The concentrations of reference toxicant solutions (RT1 and RT2) may be different; this still needs to be determined.

5. The increased tendency of *D. magna* to float on the surface of the water in test chambers needs to be understood and resolved so the test methodology may avoid floating.