A SIMPLIFIED ACUTE TOXICITY TESTING PROTOCOL With *CERIODAPHNIA DUBIA*

Prepared for:

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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