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Environmental Sciences Division Toxicology Laboratory Standard Operating Procedures

L. A. Kszos
A. J. Stewart
L. F. Wicker
G. M. Logsdon

Environmental Sciences Division
Publication No. 3337

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ENVIRONMENTAL SCIENCES DIVISION
TOXICOLOGY LABORATORY
STANDARD OPERATING PROCEDURES

L. A. Kszos
A. J. Stewart
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G. M. Logsdon

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SUBJECT: **PREFACE**

The Environmental Sciences Division's Toxicology Laboratory began conducting chronic and acute toxicity tests with fathead minnow larvae (*Pimephales promelas*) and a daphnid (*Ceriodaphnia dubia*) in October 1985. Toxicity tests with these two species, approved by the U.S. Environmental Protection Agency, were initially required by the Tennessee Department of Health and Environment in the National Pollutant Discharge Elimination System (NPDES) permit issued to the Oak Ridge Y-12 Plant on May 24, 1985, and later, in the NPDES permit issued to the Oak Ridge National Laboratory on April 1, 1986. Toxicity tests were also required in the NPDES permit modification issued to the Oak Ridge Gaseous Diffusion Plant on September 11, 1986. As we gained experience in performing the tests, it became clear that the test methods recommended by EPA were in some cases inappropriate, unsatisfactory, or ambiguous. Yet careful documentation of the test procedures and test results was necessary for legal, scientific, and fiscal reasons. This document was developed to assist the Toxicology Laboratory in its efforts to consistently provide high-quality toxicity tests. Because high-quality tests can result only if good methods are followed consistently, the document was developed first and foremost as a "working document" for use by those conducting the tests. To meet this objective, the document includes an extensive index, cross-references procedures and sections whenever appropriate, and has been thoroughly edited to ensure clarity. This document is subject to revision as new and/or improved operating procedures are developed. Please contact the senior author for recent revisions.

We thank Karen N. Gibson for her work on the design and format of this document. We also thank the technical staff of the Toxicology Laboratory, without whose support and assistance this document could not have been completed: Richard Bailey, Peggy Braden, Linda Ewald, G. Jayne Haynes, Gail Morris, and Jackie Richmond.

SUBJECT: **EXECUTIVE SUMMARY**

This document was developed to provide the personnel working in the Environmental Sciences Division's Toxicology Laboratory with documented methods for conducting toxicity tests. The document consists of two parts. The first part includes the standard operating procedures (SOPs) that are used by the laboratory in conducting toxicity tests. The second part includes reference procedures from the U.S. Environmental Protection Agency document entitled *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, upon which the Toxicology Laboratory's SOPs are based. Five of the SOPs relate specifically to the *Ceriodaphnia* survival and reproduction test. These SOPs include procedures for preparing *Ceriodaphnia* food (SOP-3), maintaining *Ceriodaphnia* cultures (SOP-4), conducting the toxicity test (SOP-13), analyzing the test data (SOP-13), and conducting a *Ceriodaphnia* reference test (SOP-15). Five additional SOPs relate specifically to the fathead minnow (*Pimephales promelas*) larval survival and growth test: methods for preparing fathead minnow larvae food (SOP-5), maintaining fathead minnow cultures (SOP-6), conducting the toxicity test (SOP-9), analyzing the test data (SOP-12), and conducting a fathead minnow reference test (SOP-14). The six remaining SOPs describe methods that are used with either or both tests: preparation of control/dilution water (SOP-1), washing of glassware (SOP-2), collection and handling of samples (SOP-7), preparation of samples (SOP-8), performance of chemical analyses (SOP-11), and data logging and care of technical notebooks (SOP-16).

SUBJECT: **ABBREVIATIONS AND CODES**

ANOVA	analysis of variance
CONC	Test concentration
CONT	control
D.F.	degrees of freedom
D.O.	dissolved oxygen
EPA	U.S. Environmental Protection Agency
ESD	Environmental Sciences Division
FC	free chlorine
FHM	fathead minnow
GLM	general linear models
ICP	Inductively Coupled Plasma Spectrophotometry
LSD	least significant difference
MSE	mean square error
ORNL	Oak Ridge National Laboratory
ORGDP	Oak Ridge Gaseous Diffusion Plant
REF	reference procedure
REP	replicate
SAS	Statistical Analysis System, registered trademark of the SAS Institute, Inc., Cary, North Carolina
SOP	standard operating procedure
SOR	siphoned out and returned
TCA	trout chow and Cerophyl mixture supplemented with algae

SUBJECT: **ABBREVIATIONS AND CODES**

TCO	trout chow and Cerophyl mixture
TRC	total residual chlorine
S	standard deviation

SUBJECT: **QUALITY ASSURANCE PLAN--CAPTIONS**

Exhibit SOP-14.1 Data sheet for fathead minnow reference test.

Exhibit SOP-15.1 Data sheet for *Ceriodaphnia* reference test.

SUBJECT: **STANDARD OPERATING PROCEDURES–CONTENTS**

The following standard operating procedures were designed specifically for the Toxicology Laboratory. Full descriptions of the individual procedures are contained in this section of the manual.

SOP-1: PREPARATION OF DILUTED DEGASSED MINERAL WATER

SOP-2: WASHING OF GLASSWARE

SOP-3: PREPARATION OF *CERIODAPHNIA* FOOD

SOP-4: MAINTENANCE OF *CERIODAPHNIA* CULTURES

SOP-5: PREPARATION OF NEWLY HATCHED BRINE SHRIMP

SOP-6: CULTURE OF FATHEAD MINNOWS

SOP-7: COLLECTION AND HANDLING OF SAMPLES

SOP-8: PREPARATION OF SAMPLES

SOP-9: FATHEAD MINNOW TOXICITY TEST

SOP-10: *CERIODAPHNIA* TOXICITY TEST

SOP-11: PERFORMANCE OF CHEMICAL ANALYSES

SOP-12: STATISTICAL ANALYSES OF DATA FROM THE FATHEAD MINNOW TOXICITY TEST

SOP-13: STATISTICAL ANALYSES OF DATA FROM THE *CERIODAPHNIA* TOXICITY TEST

SOP-14: FATHEAD MINNOW REFERENCE TEST

SOP-15: *CERIODAPHNIA* REFERENCE TEST

SOP-16: DATA LOGGING AND CARE OF TECHNICAL NOTEBOOKS

SUBJECT: **PREPARATION OF DILUTED DEGASSED MINERAL WATER**

Purpose

To provide chemically defined water for maintaining *Ceriodaphnia* cultures and for conducting fathead minnow and *Ceriodaphnia* toxicity tests.

Equipment

Mineral water (e.g., Perrier)
Deionized distilled water
Micro Algae Grow Mass Pack (Florida Aqua Farms, 5532 Old St. Joe Rd., Dade City, FL 33525)
52-L Nalgene carboy
2-L and 100-mL graduated cylinders
118-mL (8-oz.) plastic bottle
Air lines and hose
Air stones
Conductance meter (see SOP-11)
pH meter (see SOP-11)
Chlorine titrator (see SOP-11)
Chemicals for hardness and alkalinity measurements (see SOP-11)
Water preparation log sheet

Procedure

1. In a 52-L Nalgene carboy, combine mineral water and deionized distilled water in a ratio of 1:9 (5 L of mineral water plus 45 L of deionized distilled water). Use the 2-L graduated cylinder to measure the liquids.

2. Prepare the trace metals mixture (if necessary). Obtain the liquid concentrate labeled "PART A: Trace Nutrients" from the Micro Algae grow Mass Pack. Add 700 μL of the trace nutrients to a 100-mL graduated cylinder. Bring the mixture to 100 mL, using autoclaved, deionized distilled water. Transfer the 100-mL volume to a clean plastic bottle labelled "trace metals." Label the bottle with the date of preparation and initial. Store the solution in the refrigerator when it is not being used.

3. Add 500 μL of the trace metal mixture (made fresh or located in the refrigerator) per liter of water made in step 1.

4. Put an air stone on an air line, and put the air stone into the water. Then gently aerate the contents of the reservoir for 24 h. The air line should contain a carbon filter.

5. Before using the water, measure its conductivity, pH, alkalinity, and hardness, according to the directions in SOP-11. Record these measurements on the water preparation log sheet.

SUBJECT: **WASHING OF GLASSWARE**

Purpose

To clean dirty glassware.

Equipment

Alconox detergent powder
Reagent-grade nitric acid
Sodium bicarbonate
Tap water
Distilled water
Two 50-gal Nalgene mobile tubs
Rubber gloves
Protective eye glasses
Bristle brushes
Wire cart or absorbent lab cover

Procedure

Note: You should wear gloves and protective eye glasses while using nitric acid.

1. Add ~75 L of distilled water to one of the 50-gal Nalgene tubs to use as a bath. Add 7.5 L of nitric acid solution and 67.5 L of distilled water (10% nitric acid solution) to the other 50-gal Nalgene tub to make a nitric acid bath.

2. Fill one side of a double sink with a small amount (~1 Tbl.) of Alconox detergent and 8 L of warm tap water. Using a bristle brush and the warm soapy water, scrub the dirty glassware until clean. In the other half of the double sink, immediately rinse the soapy glassware with tap water. DO NOT allow the soap to dry on the glassware.

3. Soak the rinsed glassware in the nitric acid bath for at least 15 min.

4. Remove the glassware from the nitric acid bath, and rinse it in the distilled water bath. (The glassware does not have to soak in the distilled water bath.) After rinsing the glassware in the distilled water bath, rinse the glassware five times with distilled water directly from the tap.

5. Place the clean glassware upside down on a wire cart or absorbent lab cover to dry.

6. Once a week, neutralize the distilled water in the distilled water bath by adding sodium bicarbonate, and then discard it. Rinse the distilled water bath several times with distilled water; then add fresh distilled water.

7. Twice a year, neutralize the nitric acid in the nitric acid bath by adding sodium bicarbonate, and then discard it. Rinse the nitric acid bath several times with distilled water and replenish the bath (see step 1).

SUBJECT: **PREPARATION OF *CERIODAPHNIA* FOOD**

Purpose

To provide suitable food for *Ceriodaphnia* cultures and *Ceriodaphnia* toxicity tests.

Equipment

#1 Purina Trout Chow
Fleishmann's yeast
Cerophyl
Deionized distilled water
Blender
Air line and hose
Balance accurate to 0.1 g
Graduated cylinders
Three 4-L beakers
Cheesecloth
118-mL (8-oz.) plastic bottles
500-mL flask
Four 1-L flasks
4-L flask
8-L flask
2-L graduated cylinder

Procedure

1. Prepare digested trout chow as follows.

    a. In a blender, combine 13.3 g of trout chow and 2.66 L of distilled water.

    b. Mince the mixture well, and aerate it in a 4-L beaker for 7 d at 25°C.

    c. Pour the mixture into a 4-L beaker, and refrigerate it overnight.

    d. Decant the digested trout chow, and discard any particulate matter.

2. Prepare yeast as follows.

    a. In a blender, combine 10.0 g of Fleishmann's yeast and 2 L of distilled water.

    b. Blend the mixture at low speed for 5 min.

    c. Pour the supernatant into a 4-L beaker, and refrigerate it overnight.

3. Prepare Cerophyl as follows.

   a. In a blender, combine 11.1 g of Cerophyl (ground to a powder) and 2.22 L of distilled water.

   b. Blend the mixture at high speed for 5 min.

   c. Pour the mixture into a 4-L flask, add a stir bar, and place the flask on a magnetic stirrer until the end of the day. Place the mixture in the refrigerator, and allow it to settle overnight.

   d. The next day, take the mixture out of the refrigerator, and decant it. Discard any particulate matter.

4. Combine the decanted trout chow and the decanted Cerophyl. Then strain the mixture two times through four layers of cheesecloth.

5. Stir the yeast mixture, and add it to the trout chow-Cerophyl mixture (referred to as TCO).

6. Bottle the mixture in clean 118-mL (8-oz.) plastic bottles. Label the bottles with the date that the food was prepared. After labeling the bottles, freeze the food and store it in the freezer until it is needed.

7. To supplement the trout chow-Cerophyl mixture, cultivate green unicellular flagellate algae, *Haematococcus lacustris* (hereafter, *H. lacustris*), as follows:

   a. Prepare the growth medium (Moss 1972) by completing the following steps.

      (1) Make four separate stock solutions in 1-L flasks, using deionized distilled water.

         - 4.0 g/L $Ca(NO_3)_2 \bullet 4H_20$
         - 0.5 g/L K_2HPO_4
         - 2.5 g/L $MgSO_4 \bullet 7H_20$
         - 2.0 g/L $NaHCO_3$

(2) Prepare a trace element mixture by combining the following in a 500-mL flask.

- 1.5 g/L EDTA, tetrasodium salt
- 40 mg/L $FeCl_3 \bullet 6H_20$
- 30 mg/L $MgCl_2 \bullet 4H_20$
- 5 mg/L $ZnCl_2$
- 1 mg/L $CoCl_2 \bullet 6H_20$
- 5 mg/L $NaMoO_4 \bullet 2H_20$

(3) Cover the flasks of the stock solutions made in steps 7a(1) and (2), and store them in the dark.

(4) In an 8-L flask, add 10 mL of each stock listed in step 7a(1) and 2 mL of the trace element mixture listed in step 7a(2) to each liter of deionized distilled water to be used. This mixture constitutes the growth medium.

(5) Heat the medium by ONE of the following processes:

- Autoclave the medium for about 15 min, or
- After adding the appropriate volume of the nutrient stock solutions to the deionized distilled water, heat the medium on a hot plate to a final temperature of 80-90°C.

Heating will kill algae that may have inadvertently been introduced.

(6) Allow the medium to cool to room temperature. Then bubble it briefly (for 1-3 min.) with pure CO_2. Bubbling with CO_2 augments the supply of dissolved inorganic carbon in the medium and helps solubilize carbonates that may have precipitated from solution as the medium was heated.

b. Add *H. lacustris* cysts to fresh Moss culture medium to restart the culture. For an 8-L flask containing 7-8 L of medium, 5-10 mL of a suspension of *H. lacustris* cysts provides a sufficient inoculum. For a 1-L flask containing 300-500 mL of medium, 0.5-1.0 mL of cyst suspension is adequate. The new cultures do not need to be shaken, continually but the rate of culture development can be increased somewhat if the contents of the flasks are swirled by hand once or twice daily to disrupt diffusion barriers. Excystment will generally occur 3-4 d after a culture is started. Maintain three to four 8-L cultures at a time to ensure that a sufficient amount of algae will be available.

c. Culture the algae at room temperature. Light can be supplied by placing the culture containers either on a window sill that receives natural sunlight or in a growth chamber equipped with fluorescent lamps. Inspect the developing cultures carefully 4 or 5 d after starting them. Individual cells of *H. lacustris* are large enough to be discerned by eye; in an undisturbed culture vessel, any disturbance will cause the cells to quickly migrate downward as a descending cloud of visually discrete greenish-reddish cells. If the medium becomes a more opaque, highly turbid yellowish green, suspect contamination; if microscopic examination at 100 or 400× magnification reveals algae other than *H. lacustris*, discard the culture and start a new one, following the directions in step 5b. Cyst suspensions can be stored for 2 years or more and remain viable; they will encyst on schedule as described above.

d. In the vegetative stage, cells of *H. lacustris* are motile and therefore difficult to separate from the culture by centrifugation, filtration, etc. As the cultures age (~2 to 3 weeks), most of the cells encyst and settle to the bottom of the culture vessel. In this phase, the cells can easily be collected.

(1) Vigorously swirl the contents of an 8-L culture to dislodge cysts that may have adhered to the inner walls of the culture vessel.

(2) Allow the culture to stand undisturbed overnight so that the cysts can settle.

(3) Carefully siphon off the top 7.5 to 7.8 L of the medium, using a piece of tygon tubing. As siphoning nears completion, the culture vessel can be gently tilted so that few cysts will be lost as siphoning proceeds.

(4) Then swirl the remaining medium to fully resuspend the cysts, and combine the cyst-enriched aliquot in a 2-L graduated cylinder.

(5) Allow the cyst-enriched medium to stand undisturbed on the graduated cylinder overnight, as before.

(6) Siphon off excess supernatant the next morning to further concentrate the cysts to a final volume of ~300 mL.

Three 8-L cultures can be expected to yield 60-80 mL of settled cysts.

8. Siphon off enough of the medium overlying the cysts to leave a final volume of 300 mL (cysts plus medium). Add enough TCO food (prepared in steps 1-6) to bring the mixture to a final volume of 2 L. Cover the top of the 2-L graduated cylinder with clean parafilm. While holding the parafilm in the palm of your hand, invert the cylinder five or six times to mix the algae and TCO thoroughly. The methods described here will yield ten bottles of supplemented food (referred to as TCA). When mixing is complete, pour the mixture back into the trout chow bottles, and relabel the bottles with yellow tape, indicating the

preparation dates (TCA month/day/year; TCO month/day/year). Put your initials on the bottle so we know who made it. If the TCA will not be used immediately, freeze the bottles of TCA.

9. Unialgal cultures of *H. lacustris* can be purchased (for approximately $10) from the Culture Collection of Algae at The University of Texas at Austin. Address correspondence to Curator, Department of Botany, The University of Texas at Austin, Austin, TX 78712. Phone (512) 471-4019.

Work Cited

Moss, B. 1972. The influence of environmental factors on the distribution of freshwater algae: An experimental study. I. Introduction and the influence of calcium concentration. *Journal of Ecology* 60:917-932.

SUBJECT: **MAINTENANCE OF *CERIODAPHNIA* CULTURES**

Purpose

To maintain healthy cultures of *Ceriodaphnia*.

Equipment

Lighting system with timer
1000-mL pyrex glass beakers
Holding tank with plexiglass cover
Finger bowls (190 × 100 mm)
Polished glass tubing (2-mm bore, ~25-cm lengths)
1-mL pipette bulbs
Binocular stereomicroscope with fiber-optics lighting
Two 38.8-L (10-gal) aquariums
1000-μL volumetric pipette
Well/spring water or diluted degassed mineral water (see SOP-1)
Ceriodaphnia food (see SOP-3)
Paper towels
Registered *Ceriodaphnia* culture log book

Description of the System

The *Ceriodaphnia* culture system consists of six 1000-mL pyrex glass beakers. Each beaker contains approximately 1 L of culture water (well/spring or diluted degassed mineral water) and 15 adult *Ceriodaphnia*. The beakers are kept at room temperature. The beakers are located outside the Toxicology Laboratory in the main building space. Overhead lighting (5-14 ft-c, measured with a Rich Wetzed Protomatic light meter) is achieved through the use of cool-white or daylight fluorescent lamps.

Procedure

1. Change the *Ceriodaphnia* culture water once every 7 d as follows.

    a. Pour a *Ceriodaphnia* culture from the 1-L beaker into a large clean finger bowl.

    b. Wipe the empty culture beaker with a paper towel, and rinse the beaker with diluted degassed mineral water (once a month use acid-washed glassware; see SOP-2).

    c. Add 1000 mL of fresh diluted degassed mineral water to the clean beaker.

    d. Reestablish each culture by adding to the fresh water 60-70 healthy neonate *Ceriodaphnia* selected from those in the finger bowl. Use a polished glass tube and a 1-mL pipette bulb to select the animals. Examine the animals under the binocular stereo-microscope to determine their health before adding them to the new culture.

2. Feed each culture 0.5 mL of *Ceriodaphnia* food twice daily (once in the morning and once at the end of the working day).

3. Maintain the cultures at room temperature and at a photoperiod of 16:8 h (light:dark).

SUBJECT: **PREPARATION OF NEWLY HATCHED BRINE SHRIMP**

Purpose

To provide food for young fathead minnows in laboratory cultures and for fathead minnow larvae used in the toxicity tests.

Equipment

Brine shrimp eggs (high-yield variety from Aquarium Products, 180L Penrod Court, Glen Burnie, MD 21061).
Two 1-L separatory funnels
Sodium chloride (NaCl)
Air line
Modified vulcan cup with screen
Vulcan cup
Beakers
200-μL volumetric pipette
Dechlorinated tap water

Procedure

1. Prepare two batches of brine shrimp eggs on each day of the toxicity test.

2. In a 1-L separatory funnel, combine 500 mL of dechlorinated tap water, 25 g of NaCl, and 2.5 g of brine shrimp eggs.

3. Mix the contents of the funnel by gently swirling. Attach the air line to the bottom of the separatory funnel, and aerate the mixture for 24 h at 28°C.

4. Close stopcock, and remove the separatory funnel from the air line. Allow the hatched shrimp to settle in the funnel for 8 min.

5. Carefully release the settled shrimp by opening the stopcock of the funnel. Collect the shrimp in the modified vulcan cup with a metal screen (~100-μm mesh) glued to its base.

6. Rinse the shrimp once gently with dechlorinated tap water to remove excess salt.

7. Fill a regular vulcan cup with 145 mL of dechlorinated tap water, and nest the screened vulcan cup (which contains the brine shrimp) inside the regular vulcan cup. Then swirl the cup gently to mix the shrimp and water.

8. Using a 200-μL volumetric pipette, feed larvae in each fathead minnow test beaker 50 μL of the shrimp-water suspension. This amount contains 700 $\pm$ 100 shrimp, according to previous counts.

9. To save excess shrimp do the following.

   a. Place 500 mL of dechlorinated tap water in a separatory funnel.

   b. Add 25 g of NaCl and hatched brine shrimp.

   c. Aerate the suspension gently.

10. Hatched brine shrimp that are not used for a toxicity test can be fed to the culture larvae.

SUBJECT: **CULTURE OF FATHEAD MINNOWS**

Purpose

To maintain healthy fathead minnow cultures and to culture 1- to 5-d-old fathead minnow larvae (with a 48-h range in age) for the fathead minnow toxicity test.

Equipment

Dechlorinated tap water
Twenty-four 15-L aquariums (used as spawning tanks)
3-in. PVC pipe cut into 4-in. lengths, each of which is then split longitudinally to make spawning tiles (5 per aquarium)
Lighting system with timer
Two 2-L separatory funnels
Hatching chambers (clear plastic mice cages)
Air line and air stone
Petri dishes
Water bath with temperature control (±2°C)
Frozen brine shrimp
Tetramin flake food
Polished glass tubing (2-mm bore, ~25-cm lengths)
1-mL pipette bulb
Aquarium scraper
Vacuum pump
1-mL pipette tip
Tongs
Thermometer
Nets
Backup cultures of minnows to serve as future breeding stock
Nylon mesh scrub pads
Round petri dishes (125 mm × 65 mm)
Registered fathead minnow culture log book

Description of the System

The fathead minnow culture system consists of twenty-four 15-L spawning aquaria. Each aquarium contains male and female minnows in a ratio of 1:3 and five spawning tiles (PVC pipe cut into 4-in. lengths and then split longitudinally). Each aquaria receives a constant flow of dechlorinated tap water, and a hole drilled into the side of each aquaria maintains a constant water level. The water temperature of the culture system is maintained at 25 ± 0.5°C. Overhead lighting (16 h light and 8 h dark) is achieved through the use of cool-white or daylight fluorescent lamps.

Procedure

1. The following tasks must be performed daily.

   a. Check the temperature of two aquariums per group of spawning aquariums twice daily, and adjust the temperature if it is not 25 $\pm$ 0.5°C. Record the temperature in the registered fathead minnow log book. Maintain a photoperiod of 16:8 h (light:dark).

   b. Feed the fish in each spawning aquarium three times daily at approximately 4-h intervals during the work week and twice daily during the weekend. Alternate frozen brine shrimp and Tetramin flake food. Thaw and store the brine shrimp in the refrigerator as needed.

   c. In the registered fathead minnow culture log book, record any observed changes in the culture conditions, such as death, disease, pairing inconsistencies, restricted water flow, or changes in the photoperiod.

2. The collection of eggs involves the following steps.

   a. Using tongs, collect spawning tiles from the aquarium on Monday, Wednesday, and Friday. As the tiles are collected, record the size of the egg mass (small, medium, or large) in the registered fathead minnow culture log book. Remove the tiles that have eggs attached, and replace them with clean ones.

   b. Clean the top surface of the used spawning tiles (the exterior surfaces of the tiles can be safely wiped clean with a scrub pad) if necessary. Be careful not to disturb the eggs. Place the cleaned tiles in a hatching chamber.

   c. Using an air line and an air stone, aerate the eggs for 45-60 min.

   d. Using a rolling motion of the thumb, gently remove the eggs from the tiles, and place them in a round petri dish containing 10-20 mL of dechlorinated tap water. It is important to keep the eggs moist during this process. Discard eggs with fungus and eggs that have not been fertilized (their contents are dead white rather than black and white).

   e. Transfer the eggs from the petri dish to a 2-L separatory funnel containing about 1.4 L of dechlorinated tap water. Aerate the eggs, using an air line extending to the bottom of the separatory funnel; the end of the air line should be fitted with a 1-mL pipette tip to prevent eggs from settling below the point of air entry.

   f. After 24 h, allow the eggs to settle, and drain them into a petri dish. Again, remove any eggs with fungus. Return the good eggs to the separatory funnel containing fresh dechlorinated tap water, and aerate them an additional 24 h.

g. After additional aeration, allow the eggs to settle. Then drain them into a round petri dish. Discard unfertilized eggs and eggs with fungus. The remaining eggs should be measured in a 10-mL graduated cylinder: the volume of eggs provides an estimate of the number of larvae that can be expected to develop (2.5 mL of eggs yield ~1000 larvae).

h. Place the eggs in a hatching chamber with aeration, and leave them there until they are hatched. All larvae that are 1-5 d old may be used for a toxicity test. Extra larvae (those not used in a toxicity test) can be raised to become breeding adults.

i. The spawning tiles from which eggs were collected should be cleaned with a nylon mesh scrub pad and scalded with hot water before being reused.

3. Once a week the following tasks should be performed.

   a. Clean all hatching equipment by scrubbing and rinsing; scald all equipment with hot water. NEVER USE SOAP TO WASH HATCHING EQUIPMENT OR AQUARIUM SUPPLIES USED FOR FISH!

   b. Scrape the interior walls of the breeding aquariums and spawning tiles to rid them of algae. Vacuum out excess food, algae, and detritus residing on the bottom of the aquarium, using the pump.

4. Once a month the following task must be performed: check each spawning pair; using a net, remove and replace immature males, old males, and nonproducing females.

SUBJECT: **COLLECTION AND HANDLING OF SAMPLES**

Purpose

To collect ambient samples for toxicity tests.

Equipment

1- and 2-gal Nalgene bottles
Rubber boots and gloves
1-L Nalgene bottle
5-gal bucket and nylon rope
Hand-held electronic thermistor (±0.1°C) or a
glass-mercury thermometer (±0.5°C)
Cardboard boxes lined with red plastic bags (for "C-Zone" samples only)
Field data sheets
Registered water sample log book

Procedure

1. Collection and handling of samples involve the following steps.

    a. Label a clean 1- or 2-gal Nalgene bottle with sampling site identification.

    b. Put on rubber boots and gloves to do the sampling.

    c. At each sampling site, rinse the bottle with several hundred milliliters of water from the site before filling it.

    d. Fill the bottle by gently submersing the bottle in the stream or effluent. Be careful to stand downstream from the bottle during this procedure to avoid sampling water that contains sediments inadvertently suspended by the immersion of the bottle or by your movements.

    e. In sites where the depth of the water precludes obtaining a sample by immersing the bottle, use a smaller container (a 1-L Nalgene bottle) to dip the water from the site, and then pour it into the 2-gal bottle.

    f. Fill the sample bottle completely to reduce the exchange of gases between dissolved and gaseous phases.

    g. Samples that are difficult to obtain because of the terrain may be sampled with a 5-gal bucket tied to the end of a nylon rope. Rinse the bucket with water from the site before using it.

h. Collect samples from "C-Zone" sites in 2-gal Nalgene bottles, and transport them to the laboratory in cardboard boxes lined with red plastic bags to prevent the possible spread of contamination.

2. Recording field data involves the following steps.

a. Immediately after collecting the sample, record on a field data sheet the temperature of the water and the time of day that the sample was collected. Upon returing to the Toxicology Laboratory, transcribe the data into the registered water sample log book.

b. In the registered water sample log book, also record the names of those collecting the samples, ancillary information that may be of value later (e.g., "raining hard; Site 15 up 10-15 cm and very turbid"), and the time that the samples are delivered to the laboratory.

c. Measure the water temperature with a hand-held electronic thermistor (e.g., Cole-Palmer Model 85230-00, $\pm$0.1°C) or a glass-mercury thermometer ($\pm$0.5°C). Record the temperature in the registered water sample log book.

SUBJECT: **PREPARATION OF SAMPLES**

Purpose

To prepare water samples for toxicity tests.

Equipment

Volumetric pipettes (20-5000 μL, Rainin Pipetteman, adjustable)
Volumetric flasks (200-2000 mL in volume)
Graduated cylinders (10-2000 mL in volume)
Dilution water: one part degassed mineral water to nine parts deionized distilled water (see SOP-1)
Thermometer
Water bath with temperature control
30-μm Nitex mesh
Rubber gloves
Protective eye glasses
Registered water sample log book

Procedure

Note: You MUST wear gloves and protective eye glasses while preparing samples.

1. In the registered water sample log book, record the sampling time and the temperature of the water samples brought into the laboratory.

2. Heat or cool the water samples in a water bath, as required, to achieve a final temperature of 25 $\pm$ 0.5°C.

3. When preparing ambient water samples, filter the water through a 30-μm Nitex mesh before using it in toxicity tests.

4. If the samples were heated rapidly, aerate each sample for several minutes, using an air line with an air stone attached. The aeration will prevent supersaturation of gases in the water.

5. If dilutions of the water need to be made, use the appropriate volumetric flasks, graduated cylinders, and/or volumetric pipettes. If the same dilutions are used for the *Ceriodaphnia* and fathead minnow toxicity tests, one 2000-mL volumetric flask for each dilution is sufficient. If different dilutions are used, then use 2000-mL volumetric flasks for the fathead minnow test, and use 250-mL volumetric flasks for the *Ceriodaphnia* test. Dilutions for the fathead minnow and *Ceriodaphnia* tests are made with the dilution water.

SUBJECT: FATHEAD MINNOW TOXICITY TEST

Purpose

To measure the chronic toxicity of water samples to fathead minnows during 7-d static renewal exposures.

Equipment

Fathead minnow cultures (see SOP-6)
Water bath with temperature control
Dilution and control water: one part mineral water to nine parts deionized distilled water (see SOP-1)
Microbalance accurate to 0.0001 mg (Cahn)
Microweight pans (e.g., Cahn)
Drying oven
10- and 600-mL beakers
250-mL glass beaker or a crystallizing dish
Volumetric flasks
Graduated cylinders
Volumetric pipettes
Various colored stickers
Polished glass tubing (2-mm ID)
1-mL pipette bulbs
Siphoning hose (hose and modified Y-shaped tygon connector)
White plastic photographic tray
20-mL polystyrene beaker
Newly hatched brine shrimp (see SOP-5)
Perforated foil
Desiccator
Scintillation vial
Registered fathead minnow log book

Procedure

1. The following tasks should be performed in preparation for the test.

   a. Prepare the glassware. Label and color code four 600-mL beakers per test concentration (or site). Each of these four will be replicates (REPs). Label the necessary graduated cylinders and volumetric flasks.

   b. Prepare the weight beakers for the test larvae. Label 10-mL beakers with the sample, dilution, and REP number. Predry microweight pans in an 80°C oven for at least 2 h. Place a microweight pan in each 10-mL beaker, and put the beakers in a suitable container (e.g., a 250-mL glass beaker or a crystallizing dish, depending upon the number of 10-mL beakers needed). Weigh the microweight pan from each beaker on a calibrated

Cahn microbalance, and record the initial weight of each pan in the registered fathead minnow log book. Put each microweight pan back in its appropriate beaker. Cover the container of beakers with perforated foil, and store it in a sealed desiccator.

c. Prepare the weight beakers for the initial larvae. Label four 10-mL beakers as "initial 1," "initial 2," "initial 3," and "initial 4." These beakers will be used to find the weight of the larvae that initiate the test. Place a microweight pan in each 10-mL beaker, and weigh as described in step 1b.

d. Check the number of larvae available for the toxicity test. Only if necessary, order larvae from the EPA laboratory in Newtown (Cincinnati), Ohio. Note: If larvae must be ordered for a test, the request to in the EPA laboratory should be made no later than Monday afternoon to ensure that the larvae can arrive by Wednesday by Federal Express.

e. Prepare brine shrimp according to SOP-5.

2. To start the test do the following.

a. Isolate the larvae. Using a polished glass tube and pipette bulb, transfer ten larvae that are 1-5 d old (hatched within 48 h of each other) from the hatching chamber to a 20-mL polystyrene beaker containing 5-8 mL of control water. Transfer ten larvae for every REP of the test. Also transfer ten larvae for each initial beaker.

b. Begin the test. Randomly transfer larvae from the 20-mL polystyrene beakers to 600-mL beakers containing about 250 mL of test solution. The amount of water transferred with the larvae should be kept to a minimum to avoid dilution of the test solution. Randomly arrange test beakers in a temperature-controlled water bath (25 $\pm$ 0.5°C). Record the time at which the last test beaker receives its larvae and is placed in the water bath. Feed the larvae in each test beaker 50 μL of brine shrimp food twice daily at a ~8-h interval (at the beginning and at the end of the work day). Record the source and hatching date of the larvae that are used. Identify the test water, the dilutions used, and the dilution water. Any other pertinent information about the test or sample should also be recorded at this time in the registered fathead minnow log book.

c. Transfer all the larvae from the initial beakers into a scintillation vial containing dechlorinated tap water. Add several drops of formaldehyde solution. The scintillation vial will now contain 40 larvae. Label the vial with "initials," the date and the name of the test. Store the vial in a safe location until the end of the test.

3. Repeat the following renewal process every day, starting 24 h after the test begins and continuing until the test is finished on Day 7.

a. Take fresh samples and make fresh dilutions.

b. Change the water of all REP beakers for a particular concentration (or site) before

starting the next four-beaker series.

(1) Siphon off old water, excess shrimp, and detritus from the beakers, using rubber tubing and a modified foot made from a Y-shaped tygon connector. Slowly siphon the water from the beaker into a white plastic photographic tray until ~50 mL of old test solution remains. Control the flow through the siphon by holding one gloved finger over the end of the tubing.

(2) If any larvae are accidentally siphoned off with the water, retrieve them from the plastic tray, using a polished glass tube and pipette bulb. Then return them to the beakers.

(3) Record the number of these larvae as SOR (siphoned out and returned) in the registered fathead minnow log book. Record the number of all surviving larvae in each beaker (including the SORs), and discard the dead larvae.

(4) Add ~250 mL of fresh sample to each beaker. Add the fresh solution very slowly, gently pouring it down the side of the beaker to avoid disturbing the larvae.

c. Feed the larvae one drop of brine shrimp twice daily; record the temperature within representative beakers and within the water bath with the morning feeding.

4. Terminate the test after the fish have been exposed to the test solution for 7 consecutive days.

a. Siphon out as much of the test solution as possible, and pour the last amount of solution (containing the surviving larvae) through a net.

b. Kill the larvae that are retained on the net by severing their spinal cords with forceps. Remove each larva from the net with forceps, and place all larvae from a REP beaker on the microweight pan corresponding to the beaker from which they were taken.

c. Obtain the scintillation vial containing the initial larvae. Pour the contents of the vial into a net to obtain the larvae. You may need to rinse the vial several times with distilled water to remove all the larvae. After all the larvae are in the net, rinse the larvae several time to remove the formaldehyde solution. Remove ten larvae from the net and place on a microweight pan from an initial beaker. Repeat this procedure for all four initial beakers.

d. Put each pan in its labeled 10-mL beaker, and place the beakers in a suitable container.

e. Cover the container with perforated foil, and let the contents dry overnight at 80°C in a drying oven.

f. The following morning, cool the larvae in a desiccator before weighing them with a

calibrated Cahn microbalance.

g. Record the final pan weights in the registered fathead minnow log book.

5. Prepare the data by making the following calculations.

a. Calculate the percent survival of larvae in each REP as follows:

$$\% \text{ survival} = 100 \times \frac{\text{number of larvae surviving 7 d}}{\text{number of larvae at the start of the test}}.$$

b. Calculate the mean percent survival of larvae in each concentration (with four REPs) as follows:

$$\text{mean } \% \text{ survival} = \frac{\%\text{ survival REP 1} + \%\text{ survival REP 2} + \%\text{ survival REP 3} + \%\text{ survival REP 4}}{4}.$$

c. Subtract the weight of the microweight pan from the weight of the microweight pan plus the larvae. This value is the final larvae weight.

d. Calculate the mean initial larvae weight. For each initial beaker, subtract the weight of the microweight pan from the weight of the microweight pan plus the larvae for each pan. Calculate the mean initial larvae weight as follows:

$$\text{mean initial larvae weight} = \frac{\text{weight initial 1} + \text{weight initial 2} + \text{weight initial 3} + \text{weight initial 4}}{4}.$$

e. Calculate larval growth for each REP simply as the mean change in weight per larva:

$$\text{growth per larva} = \frac{\text{final larvae weight - mean initial larvae weight}}{\text{number of surviving larvae}}.$$

f. Calculate the mean growth for each concentration or site

$$\text{mean growth} = \frac{\text{growth (REP 1)} + \ldots + \text{growth (REP } n\text{)}}{n},$$

where n = the number of REPs used in the computation.

Note: A SAS program in the AQS27558 area is available to calculate mean growth per larvae. The program requires as input the weight of the empty foil pan, the weight of the foil pan after the fish have been dried upon it, and the mean weight of the larvae at the start of the test.

SUBJECT: *CERIODAPHNIA* TOXICITY TEST

Purpose

To measure the chronic toxicity of ambient or waste waters to *Ceriodaphnia* in 7-d static renewal exposures.

Equipment

Ceriodaphnia cultures (see SOP-4)
Water bath with temperature control
Dilution and control water: one part mineral water to nine parts deionized distilled water (see SOP-1)
Binocular stereomicroscope
Magnifying lamp
Large finger bowls (190 × 100 mm)
Small finger bowls (70 mm × 50 mm)
20-mL polystyrene cups
Polished glass tubing (2-mm ID)
1-mL pipette bulbs
Disposable pasteur pipettes
Styrofoam racks for test vessels
Thermometer
Ceriodaphnia food (see SOP-3)
Hematococcus lacustris cultures (see SOP-3)
Volumetric flasks
Graduated cylinders
100-μL fixed- or adjustable-volume pipette
Stir bar
Magnetic stirrer
Stage micrometer
Registered *Ceriodaphnia* log book

Procedure

1. In preparation for the test, complete the following steps.

   a. Prepare the glassware. Label 20-mL polystyrene beakers with sample, dilution, and replicate (REP) number (ten REPs are typically used per concentration or site; additional REPs may be required for some tests). Label the necessary volumetric flasks, graduated cylinders, and beakers.

   b. Obtain neonates for the test. Before isolating adults, complete water chemistry measurements (pH, conductivity, alkalinity, and hardness) of the control water. Three days before the start of a test, isolate adult females from healthy set-up cultures maintained in the laboratory. Using a polished 2-mm (ID) glass tube fitted with a pipette bulb, remove 60-70 adults from the set-up cultures and place them in a large fingerbowl

containing the control water. If cared for properly, these adults will produce approximately 40-50 neonates for the test. Use as many fingerbowls (and adults) as necessary to produce the desired number of neonates (which depends upon the size of the test). Each day following isolation, remove neonates from the fingerbowls and discard them, without changing the water. Feed the isolated cultures 2 mL of food twice daily (once in the morning and once at the end of the workday). Maintain the isolates at 25 $\pm$ 0.5°C.

2. To perform the test do the following.

   a. Isolate neonates. The neonates used for the test must be less than 24-h old and be born within a 6-h period. If the test will not be set up until late afternoon, begin the collection procedure at an appropriate time on the day before to ensure that the young collected will not be older than 24 h.

      (1) The morning of the day before the test is to start, remove the young from the isolated cultures, and set them aside for the preparation of the next week's test.

      (2) Within the next 6 h, collect all the young necessary for the test, using a pasteur pipette and bulb and, if necessary, a magnifying lamp.

      (3) Place the isolated neonates in a small finger bowl containing the control water that will be used in the test.

      (4) Feed the neonates 0.75 mL of *Ceriodaphnia* food.

   b. Start the test.

      (1) Set up labeled beakers for the test, with ten REPs per concentration.

      (2) Using the 100-μL fixed- or adjustable-volume pipette, add 100 μL of food and 15-17 mL of test solution to each beaker. Stir the *Ceriodaphnia* food gently with a stir bar and a magnetic stirrer as the food is used.

      (3) Include a control with the appropriate dilution water in every test.

      (4) Place one neonate (collected the day before) in each test beaker, using a pasteur pipette.

      (5) Place the beakers in styrofoam racks, and put the racks in the temperature-controlled water bath.

      (6) In the registered *Ceriodaphnia* log book, record the starting time. Also record the age of the neonates used (e.g., 0-6 h) and the time they were isolated. Using the batch-specific codes, identify the dilution water and the food. Also record the dilutions for each coded test and any additional pertinent information.

3. Perform a 24-h renewal each day during the 7-d test period.

   a. Prepare fresh dilutions each day.

   b. Using a polished glass tube, a pipette bulb, and a microscope if necessary, transfer each animal daily to a new test beaker containing 15-17 mL of freshly prepared test solution and 100 μL of *Ceriodaphnia* food.

   c. Record daily the survival, the number of young produced (if any), the time that the transfer was made, and the temperature of the water (both in the water bath and in representative beakers).

   d. Discard both the neonates produced by the test animals and the old test solution.

   e. On the final day of the test, discard adults and young along with the old test solution.

   f. Code males appropriately if they are present. (For some tests, the 7-d old animals can be measured with a stage micrometer to determine their length before they are discarded.)

   g. The test should be terminated 7 d $\pm$ 2 h after being started.

4. Prepare the data by making the following calculations.

   a. Calculate the mean fecundity of the *Ceriodaphnia* in each concentration. Various procedures can be used to compute reductions in the fecundity of the test animals. The following procedure excludes males from the analysis and includes only females (and their offspring) that survive all 7 d of the test. This method is deliberately conservative (n is often lower than 10) and is statistically wasteful (it excludes offspring of females that died before 7 d); however, it is advantageous in that it unambiguously distinguishes the effects of the toxicants on the fecundity and endpoint from the effects on the mortality endpoint.

$$\text{fecundity} = \frac{\text{total number of young produced by surviving females}}{\text{number of females surviving the 7-d test period}}.$$

   b. Calculate the standard deviation (S) for the mean fecundity value. This calculation is easily accomplished by using a calculator with statistical capability.

   c. Calculate the percent survival in each concentration by using the following equation.

$$\%\ \text{survival} = 100 \times \frac{\text{number of animals that survived the 7-d test}}{\text{number of neonates used to start the test}}.$$

SUBJECT: **PERFORMANCE OF CHEMICAL ANALYSES**

Purpose

To analyze pH, alkalinity, conductivity, hardness, and total residual and free chlorine in all water samples daily and to measure the dissolved oxygen in samples during the fathead minnow test.

Equipment

pH meter (Orion model 811) equipped with a Fisher (13-639-80) probe
Conductance meter (YSI model 32) with Pyrex conductance cell (K = 1.0/cm) and temperature probe (YSI model 3220)
Chlorine titrator (Wallace and Tiernan)
Dissolved oxygen meter (VWR) with submersible probe
Betz hardness titrating solution
Eriochrome Black T
Sodium chloride (NaCl)
Calgon certified hardness buffer
Phenylarsene oxide
Potassium iodide
Stir bars
Magnetic stirrer
pH buffers (4.00, 7.00, and 10.00)
1 *N* HCl
Two 25-mL burettes
100-mL beakers
50-mL graduated cylinder
Pasteur pipettes
1-mL pipette bulbs
20-mL polystyrene cups
8-oz. specimen containers
Kimwipes or facial tissues
Potassium iodide
Deionized distilled water
Registered chemistry log book

Procedure

1. Determine the pH as follows.

   a. Use the pH meter and probe to measure the pH of the samples. Gently stir the samples with a stir bar and a magnetic stirrer while measuring.

   b. Each day before making pH measurements, calibrate the meter with pH 4.00 ($\pm$0.01) and 10.0 ($\pm$0.01) buffers (traceable to National Bureau of Standards Reference Standards; Red Bird Service, Inc.).

(1) Fill one 20-mL polystyrene cup with the pH 4.00 buffer and another cup with the 10.0 buffer. Slide the plastic sleeve on the probe down to expose the vent hole of the probe. Rinse the probe with distilled water and gently wipe off excess liquid with a Kimwipe (or facial tissue). For quality control reasons, it is important to record the pH measurement before and after calibration. Submerge the probe in the pH 4.00 buffer, and move the function knob of the meter from "standby" to 4.00. Record the pH as "before" in the registered chemistry log book.

(2) Repeat the procedure in step 1b(1) with the pH 10.00 buffer, but turn the function knob to 10.00.

(3) Now calibrate the meter as follows. Submerge the probe in the pH 4.00 buffer, and move the function knob of the meter from "standby" to 4.00. Push the calibration button until it reads "...". When the calibration button remains lighted, push the button again. The meter should read 4.00 ($\pm$0.02). Record the pH reading as "after" in the registered chemistry log book.

(4) Repeat the procedure in step 1b(3) with the pH 10.00 buffer and the function knob adjusted to 10.00.

(5) After the probe has been calibrated, check the probe's response by moving the selection knob to "slope." If the value is less than 95%, attempt to recalibrate with the two buffers as described, or clean or replace the probe as required.

c. Measure the pH of each water sample by submerging the probe in 50 mL of water. Always rinse the probe with deionized distilled water after measuring each sample. Record the pH value directly from the meter into the registered chemistry log book. Also record the number of the pH meter that you are using.

d. Always return the function knob to "standby" when finished, and slide the protective sleeve upwards to cover the vent hole of the probe. Store the pH probe immersed in pH 7.00 buffer.

2. Determine the alkalinity as follows.

a. Alkalinity is a measure of a water sample's capacity to neutralize a strong acid. Alkalinity is determined by titrating 50-mL samples with 0.01 *N* HCl (prepared from 1 *N* HCl stock) to a potentiometric endpoint of 4.50 $\pm$ 0.04. Gently stir the sample with a stir bar and magnetic stirrer during the titration.

b. Submerge the pH probe [calibrated as in steps 1b(3) and b(4)] in the 50-mL sample. Fill the 25-mL burette (graded into 0.1-mL units, readable to the nearest 0.05 mL) with the titrant (0.01 *N* HCl).

c. Allow the pH to stabilize (this step can be used to measure pH as in step 1c above). Slowly add the titrant (0.01 *N* HCl) until the sample reaches an endpoint of 4.50 $\pm$ 0.04. If a full-strength sample is being analyzed, calculate the amount of alkalinity (expressed as milligrams of $CaCO_3$ per liter) by multiplying the milliliters of consumed acid solution (see the reading on the burette) by 10 (e.g., 10.5 mL of 0.1 *N* HCl $\times$ 10 = 105.0 mg $CaCO_3$ per L).

d. If the amount of titrant needed to reach pH 4.50 is greater than 25 mL (in rare instances), a 1:2 dilution of the sample is necessary. In a 50-mL graduated cylinder, add 25 mL of full-strength sample, and bring it to volume with deionized distilled water. Titrate as in step 2c. Calculate the amount of alkalinity in milligrams $CaCO_3$ per liter, by multiplying the milliliters of consumed acid solution by 20.

e. Record the amount of alkalinity (expressed as mg of $CaCO_3$ per liter, as calculated above) directly into the registered chemistry log book. If the sample is diluted, enter a small capital "D" next to the alkalinity value. Also record the number of the pH meter you are using.

3. Determine the hardness as follows.

a. Hardness is a measure of divalent cations (primarily Ca^{2+} and Mg^{2+}) per unit volume of water. As in the measurement of alkalinity, hardness is expressed in terms of the amount of $CaCO_3$ (mg/L) that it would take to yield a particular amount of hardness. Determine hardness by titrating 50-mL water samples with Betz hardness titrating solution (1 mL of which is equivalent to 1 mg of hardness) to a colorimetric endpoint. Use Eriochrome Black T, which changes color when complexed with Ca^{2+} and Mg^{2+}, to determine when all hardness ions have been removed by the titrant. Stir the sample with a stir bar and magnetic stirrer during titration.

b. Submerge the pH probe (calibrated as in step 1b) into the 50-mL sample. Fill a 25-mL burette (graded into 0.1-mL units and readable to the nearest 0.05 mL) with the Betz hardness titrating solution.

c. Allow the pH to stabilize. Adjust the pH of the sample to 10.0, adding Calgon certified hardness buffer one drop at a time and adding 0.05 g (a "pinch") of the indicator (0.5 g Eriochrome Black T mixed with 100 g NaCl).

d. Slowly add Betz hardness titrating solution until the color of the sample changes from red to purple. At this point, add the Betz hardness titrating solution one drop at a time until the color of the sample changes from purple to blue. If a full-strength sample is being analyzed, calculate the amount of hardness, expressed as milligrams of $CaCO_3$ per liter, by multiplying the milliliters of titrant by 20 (e.g., hardness is 10.5 mL of titrant $\times$ 20 = 210 mg $CaCO_3$ per L).

e. If the amount of titrating solution needed to reach the colorimetric endpoint (blue) is greater than 15 mL, a 1:2 or 1:5 dilution of the sample is necessary.

(1) To make a 1:2 dilution, in a 50-mL graduated cylinder, add 25 mL of full-strength sample, and bring it to volume with deionized distilled water. Titrate as in step 3d. Calculate the amount of hardness, expressed as milligrams of $CaCO_3$, by multiplying the milliliters of titrant by 40 (e.g., 12 mL of titrant × 40 = 480).

(2) To make a 1:5 dilution, in a 50-mL graduated cylinder, add 10 mL of full-strength sample, and bring it to volume with deionized distilled water. Titrate as in step 3d. Calculate the amount of hardness, expressed as milligrams of $CaCO_3$, by multiplying the milliliters of titrant by 100.

f. In the registered chemistry log book, record the amount of hardness in milligrams of $CaCO_3$ (as calculated). Also record the number of the pH meter you are using. If the sample is diluted, place a small capital "D" next to the hardness value; use a subscript 2 or 5 to indicate a 1:2 or 1:5 dilution, respectively.

4. Determine the conductivity as follows.

a. Measure the conductivity of each water sample with the conductance meter equipped with the conductance cell and probe. Evaluate the responses of each newly installed cell for at least ten temperatures ranging from 1°C to 40°C to determine the appropriate compensation setting for correct temperature compensation. Subsequent conductance readings are then standardized to 25°C electronically. Evaluate the response of the meter and cell periodically for sustained linearity by analyzing dilutions of various test solutions. Replatinize the conductance cell as required, and check it for thermal response after replatination.

b. Fill a suitable container (e.g., 8-oz. specimen container) with a water sample. The depth of the water must be at least 8 cm.

c. Submerge the conductance cell and the temperature probe in the sample; carefully raise and lower the cell and probe several times to ensure that the sensors are exposed to a well-mixed sample and to force any air out of the probe via the vent of the probe. Move the function knob to "Temperature Coefficient," and adjust the range knob to the proper setting. Between samples, rinse the cell and probe with deionized distilled water.

d. Record the conductivity values directly from the meter into the registered chemistry log book. Record the number of the conductance meter, conductance cell, and temperature probe that you are using.

e. Always return the function knob to "off" when the meter is not in use.

5. Measure the amount of total residual chlorine (TRC) and free chlorine (FC) as follows.

   a. Measure TRC and FC amperometrically with the chlorine titrator. Use phenylarsene oxide as the titrant.

   b. Always titrate samples to be analyzed for TRC or FC immediately upon their arrival at the laboratory because of the rapidity with which chlorine species are converted to chloride.

   c. Fill the sample vessel with 200 mL of water (to the white line). Measure TRC by adding 1 mL of 4.0 pH buffer and 1.0 mL of potassium iodide to 200 mL of the sample before titrating. Measure FC by adding 1 mL of 7.0 pH buffer to 200 mL of sample before titrating. Note: The buffers (4.0 and 7.0) may not be adequate to control pH to the desired value for some samples. It may be necessary to use H_2SO_4 to adjust the pH of such samples close to 4.0 with (for TRC) or to 7.0 (for FC) before adding the appropriate buffer.

   d. Adjust the range of the chlorine titrator to read approximately 80-100. Wait for the needle to stabilize. Slowly add the titrant (~0.01 mL at a time) until an addition of titrant no longer causes the needle to move. As titration proceeds, readjust the range upward to 80-100 if the needle falls below 10.

   e. The amount of titrant used is equal to the amount of TRC (or FC) in mg/L. Read the amount of titrant directly from the burette, and record the amount in the registered chemistry log book. Also record the number of the titrator you are using.

6. Determine the amount of dissolved oxygen (DO) for water samples and for the fathead minnow test as follows.

   a. DO is routinely measured with a VWR meter equipped with a submersible probe.

   b. Measure DO both in the fresh water samples taken daily ("new water") and in the fathead minnow beakers after 24 h ("old water"). Typically, one beaker per replicate is measured.

   c. Calibrate the meter before each use as follows. Remove the probe from the case and turn the function knob of the meter to "Air Cal" (for "air calibration"). Allow the reading to stabilize. Turn the calibration knob until the meter reads 20.2. Record the temperature and the DO at this setting by turning the function knob to the appropriate setting. Record this information in the registered water chemistry log book as "DO Calibration Before."

   d. Measure the DO in each sample by setting the function knob of the meter to "DO" and submerging the probe in the water sample. Record the DO of each sample, measured in mg/L, directly from the meter to the registered chemistry log book. Also record the number of the DO meter and probe you are using.

e. After all of the DO measurements have been taken, read the air calibration, temperature, and DO as in step 6b above. Record this information in the registered chemistry log book as "DO Calibration After."

f. Always return the function knob to "standby" when the meter is not in use.

SUBJECT: **STATISTICAL ANALYSES OF DATA FROM THE FATHEAD MINNOW TOXICITY TEST**

Purpose

To provide final statistical analyses of fathead minnow survival and growth for the toxicity test report after the data sets have been analyzed by the General Linear Models (GLM) Procedure.

Procedure

1. Analyze the survival data.

   Note: If mean survival in all concentrations and in the control is ≥80%, then the GLM analysis is not necessary, and the following statement should be included in the final toxicity test report: "No statistical analyses were conducted, for survival was ≥80% in all concentrations that were tested." Otherwise, the following steps apply.

   a. Obtain output of the GLM table from the computer by using a SAS program in the ENVSCLAQS27558 area. The following is an example of a GLM table from the SAS program.

General Linear Models Procedure

Dependent Variable: SURV

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4400.1237743	1466.7079248	31.72	0.0001
Error	12	554.8067384	46.2338949		
Corrected Total	15	4954.9305127			

R-Square	C.V.	Root MSE	SURV Mean
0.888029	9.7539530	6.7995511	69.71072249

   Note: "Pr" signifies probability.

   b. Transcribe the information directly from the SAS-generated GLM table to the GLM table in the final toxicity test report. The following is an example of the format of the final GLM table.

GLM analysis of fathead minnow survival

Source	D.F.	Sum of squares	Mean square	Calculated F	F (0.05)
Among	3	4400.124	1466.708	31.72	3.49
Within	12	554.807	46.234		
Total	15	4954.931		(Pr > F = 0.0001)	

The following pairs of terms from the two GLM tables are synonymous: "model"/"among," "error"/"within," "corrected total"/"total," and "F value"/ "calculated F." Directly transcribe the degrees of freedom (D.F.), the sum of squares, and the mean square for the model and the error. Also transcribe the total D.F. and the total sum of squares, as well as the F value and the Pr>F value.

c. Find $F(0.05)$ for the GLM table in the final toxicity test report. Each GLM table in the final report needs to include this value. Use a "Values for F" table in a statistical book (e.g., Steel and Torrie 1960, p. 436). Find the F value for alpha = 0.05 by using the D.F. from the "among" row in the GLM table as the numerator *df* and the D.F. from the "within" row as the denominator *df*. Enter the F value under the $F(0.05)$ column in the GLM table to complete the table.

Example:

In the GLM table in step 1b, the numerator *df* is 3 and the denominator *df* is 12; therefore; the F value is 3.49.

d. Compute the least significant difference (LSD). The LSD is used to determine whether or not the fish in a particular concentration or site had significantly lower survival than the control. It is the minimum amount of survival that can be statistically determined from the data in the GLM table. The calculations are used to complete the following sentence in the final toxicity test report: "For this set of data, the least significant difference is __._ (arcsin transformed). T_d = _.__, and p = 0.05 (one-tailed test)."

(1) Because survival data are expressed as a percentage, the data are "bound": that is, they cannot be higher than 100 or lower than 0. Because of the "boundedness," the survival values must first be transformed (by arcsin square root).

The SAS program which prints out the GLM table also prints out the mean transformed survival value for each concentration (or site) and for the control. Alternatively, the transformed value for each replicate (REP) in the control can be found by using the appropriate table in a statistical book (e.g., Steel and Torrie 1960, p. 448).

Example:

> If mean survival of the fathead minnows in REP 1 of the controls is 90%, its transformed value would be 71.56. If the mean survival in REP 2 is 80%, its corresponding transformed value would be 63.44.

Using all four transformed values, calculate the mean transformed survival value:

$$\text{mean}_t = \frac{(\text{REP 1})_t + (\text{REP 2})_t + (\text{REP 3})_t + (\text{REP 4})_t}{4},$$

where t indicates an arcsin square root transformation.

(2) When you have the transformed survival values, compute the LSD by using the following equation:

$$\text{LSD} = t_d \times \sqrt{\frac{2 \times \text{MSE}}{4}},$$

where MSE is the mean square error and t_d is Dunnett's t-value at 0.05.

Dunnett's t (0.05) can be found in a statistical book (e.g., Steel and Torrie 1960, p. 446). Use the alpha and D.F. values you used to find F in 1c, but in this case the "among" D.F. value is p (the number of treatment means excluding the control) and the "within" D.F. value is the error D.F. value along the left-hand side of the table. As in step 1c, alpha is 0.05. Dunnett's t-value is found at the intersection of the p column and the error row. MSE can be found on the GLM table: it is the mean square value in the "within" row.

Example:

> In the GLM table given in step 1b, the "among" D.F. value is 3 and the "within" D.F. value is 12; therefore, Dunnett's t (0.05) is 2.29. The MSE is 46.234. Computed from the LSD equation in step 1d, the LSD is 11.01.

e. Compute the LSD expressed as a percentage of the control. This information will be used to complete the following sentence in the final toxicity test report: "This represents a __._% reduction in survival."

To calculate the LSD expressed as a percentage of the control, use the following equation:

$$\text{LSD as a \% reduction} = 100 \times \frac{\text{LSD (transformed data)}}{\text{mean survival of the control (transformed)}}.$$

Example:

From the data in step 1f and the LSD calculation in step 1d(2), the LSD is 11.01 and the mean survival of the control (transformed) is 90; therefore, the LSD expressed as a percentage of the control is 12.2%.

f. Determine if the fish in a particular concentration (or site) had significantly lower survival than the control. This analysis is conducted only when the Pr > *F* value in the GLM table is $\leq$0.05. This analysis will determine where the asterisks are to be added to the following table in the final toxicity test report.

Survival

Effluent dilution	Replicate 1	2	3	4	Mean
Control	100	100	100	100	100.0
100%	50	40	60	70	55.0*
50%	70	80	80	70	75.0*
25%	90	100	100	90	95.0

Note: Survival values in the summary table for fathead minnow survival are in nontransformed values.

Obtain the transformed mean survival values for each concentration and the control (as in step 1d). Subtract the LSD from the transformed mean survival of the control. If any transformed mean survival value of a concentration is lower than this value, it should receive an asterisk.

Example:

With the data in the table above, the arcsin transformed survival values are as follows: control = 90; 100% = 47.9; 50% = 60.11; and 25% = 80.78. If the LSD is 11.01, then the arcsine transformed value of the control (90) minus the LSD is 78.99. Because the arcsine transformed survival values for fish in the 50% and 100% concentrations are less than 78.99, place an asterisk next to the mean survival value in the table as shown.

2. Analyze the growth data.

a. Obtain output of the GLM table from the computer by using a SAS program in the ENVSCI.AQS27558 area. The following is an example of a GLM table from the SAS program.

General Linear Models Procedure

Dependent Variable: WT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.24803625	0.08267875	13.52	0.0004
Error	12	0.07338550	0.00611546		
Corrected Total	15	0.32142175			

R-Square	C.V.	Root MSE	WT Mean
0.771685	18.872132	0.07820140	0.41437500

Note: "Pr" signifies probability.

b. Transcribe the information directly from the SAS-generated GLM table to the GLM table in the final toxicity test report. The following is an example of the format of the final GLM table.

GLM analysis of fathead minnow growth

Source	D.F. squares	Sum of square	Mean F	Calculated	$F(0.05)$
Among	3	0.2480	0.0827	13.52	3.49
Within	12	0.0734	0.0061		
Total 15	0.3214		(Pr > F = 0.0004)		

The following pairs of terms from the two GLM tables are synonymous: "model"/"among," "error"/"within," "corrected total"/"total," and "F value/ "calculated F." Directly transcribe the degrees of freedom (D.F.), the sum of squares, and the mean square for the model and error. Also transcribe the total D.F. and the total sum of squares, as well as the F value and the Pr>F value.

c. Find $F(0.05)$ for the GLM table in the final toxicity test report. Each GLM table in the final report needs to include this value. Use a "Values for F" table in a statistical book (e.g., Steel and Torrie 1960, p. 436). Find the F value for alpha = 0.05 by using the D.F. from the "among" row in the GLM table as the numerator *df* and the D.F. from the "within" row as the denominator *df*. Enter the F value under the $F(0.05)$ column in the GLM table to complete the table.

Example:

In the GLM table given in step 2b, the "among" D.F. value is 3 and the "within" D.F. value is 12; therefore, the F value is 3.49.

d. Compute the LSD. The LSD is used to determine whether or not the fish in a particular concentration or site had significantly lower growth than the control. It is the minimum amount of growth that can be statistically determined from the data in the GLM table. The calculations are used to complete the following sentence in the final toxicity test report: "No transformation was used before data analysis. For this set of data, the least significant difference is _.___ mg. T_d = _.__, and p = 0.05 (one-tailed test)."

Compute the LSD by using the following equation:

$$\text{LSD} = t_d \times \sqrt{\frac{2 \times \text{MSE}}{____}},$$

Where t_d is Dunnett's t-value at 0.05 and MSE is the mean square error.

Dunnett's t can be found in a statistical book (e.g., Steel and Torrie 1960, p. 446). Use the alpha and D.F. values you used to find F in step 2c, but in this case the "among" D.F. value is p (the number of treatment means excluding the control) and the "within" D.F. value is the error D.F. value along the left-hand side of the table. As in step 2c, alpha is 0.05. Dunnett's t-value is found at the intersection of the p column and the error row. The MSE can be found on the GLM table: it is the mean square value in the "within" row.

Example:

In the GLM table given in step 2b, the "among" D.F. value is 3 and the "within" D.F. value is 12; therefore, Dunnett's t (0.05) is 2.29. The MSE is 0.0061. Computed from the LSD equation in step 2d, the LSD is 0.055.

e. Compute the LSD expressed as a percentage of the control. This information will be used to complete the following sentence in the final toxicity test report: "This represents a __._% reduction in dry weight."

To calculate the LSD expressed as a percentage of the control, use the following equation:

$$\text{LSD, as \% reduction} = 100 \times \frac{\text{LSD}}{\text{mean weight of the control}}$$

The mean weight for the control is calculated following each toxicity test (SOP-9) and can be found in the registered fathead minnow log book.

Example:

From the data in step 2f and the LSD calculated in step 2d, the LSD is 0.055 mg and the mean weight of the control is 0.449 mg; therefore, the LSD expressed as a percentage of the control is 10.7%.

f. Determine if the fish in a particular concentration (or site) had significantly lower growth than the fish in the controls. This analysis is completed only if the GLM indicates a significant difference between treatments (The Pr>F is $\leq$0.05). Subtract the LSD value calculated in step 2d from the mean weight of the controls. All mean weight values that are less than this value should receive an asterisk.

Dry weight

Effluent dilution	Replicate 1	2	3	4	Mean ± *S*
Control	0.586	0.324	0.557	0.595	0.516 ± 0.129
100%	0.201	0.204	0.204	0.202	0.202 ± 0.038*
50%	0.414	0.427	0.495	0.458	0.449 ± 0.036*
25%	0.595	0.409	0.511	0.448	0.491 ± 0.081

Example:

The mean weight of the controls is 0.516 mg, and the LSD is 0.055 mg (calculated in step 2d). The mean weight of the controls minus the LSD is 0.461 mg. Because the weight values of the fish in the 50% and 100% concentrations are less than 0.461 mg, place an asterisk beside the standard deviation of the mean weight value (see table).

Work Cited

Steele, R. G. D. and J. H. Torrie. 1960. *Principles and Procedures of Statistics, with Special Reference to the Biological Sciences.* McGraw-Hill, New York.

SUBJECT: **STATISTICAL ANALYSES OF DATA FROM THE *CERIODAPHNIA* TOXICITY TEST**

Purpose

To provide final statistical analyses for fecundity and survival of *Ceriodaphnia* after the data sets have been analyzed by the General Linear Models (GLM) Procedure.

Procedure

1. Analyze the survival data.

   a. Transcribe the mean *Ceriodaphnia* survival, the mean reproduction, and the reproduction standard deviation directly from the summary table in the registered *Ceriodaphnia* log book to the summary table in the final toxicity test report:

Summary of *Ceriodaphnia* test results

Effluent dilution	Number of replicates	Number of females surviving 7 d	Mean number of offspring per female ($\pm$ *S*)
Control	10	10	23.4 $\pm$ 2.8
5.0%	10	10	22.9 $\pm$ 2.7
10.0%	10	10	13.9 $\pm$ 2.0*
25.0%	10	4*	9.2 $\pm$ 3.9

   b. Use the Fisher's Exact Test to analyze *Ceriodaphnia* survival. This analysis will determine if *Ceriodaphnia* in a particular concentration had a significantly lower survival than the control, and it will determine where the asterisks should be added to the survival column in the table in the final toxicity test report (see step 1a).

   The following table may be used to find the critical value for Fisher's Exact Test (with ten replicates).

Mean survival in controls	Critical value
10	6
9	4
8	3
7	2

Determine the critical value for the data set. If mean survival in a concentration is equal to or less than the critical value, place an asterisk next to the survival value in that concentration. If the mean survival of the controls is 7, the test may be considered invalid.

Example:

In the table in step 1a, the mean survival of the controls is 10; therefore the critical value is 6. Because the mean survival in the 25% concentration is 4 (below the critical value), an asterisk is placed next to the survival value.

2. Analyze the fecundity (reproduction) data.

   a. Obtain output of the GLM table from the computer (produced by a SAS program in the ENVSCI.AQS27558 area). The GLM analysis for fecundity includes only those concentrations for which survival was not significantly lower than the survival in the controls (see step 1b). The following is an example of a GLM table from the SAS program.

General Linear Models Procedure

Dependent Variable: REPRO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	571.66666667	285.83333333	45.34	0.0001
Error	27	170.20000000	6.30370370		
Corrected Total	29	741.86666667			

R-Square	C.V.	Root MSE	REPRO Mean
0.770579	12.511883	2.5107178	20.06666667

Note: "Pr" signifies probability.

   b. Transcribe information directly from the SAS-generated GLM table to the GLM table in the final toxicity test report. The following is an example of the format of the final GLM table.

GLM analysis of *Ceriodaphnia* fecundity

Source	D.F.	Sum of squares	Mean square	Calculated F	$F_{(0.05)}$
Among	2	571.667	285.833	45.34	3.35
Within	27	170.200	6.304		
Total	29	741.867		(Pr > F = 0.0001)	

The following pairs of terms from the two GLM tables are synonymous: "model"/"among," "error"/"within," "corrected total"/"total," and "F value"/ "calculated F." Directly transcribe the degrees of freedom (D.F.), the sum of squares, and the mean square for the model and the error. Also transcribe the total D.F. and the total sum of squares, as well as the F value and the Pr>F value.

c. Find $F_{(0.05)}$ for the GLM table in the final toxicity test report. Each GLM table in the final report needs to include this value. Use a "Values for F" table in a statistical book (e.g., Steel and Torrie 1960, p. 436). Find the F value for alpha = 0.05 by using the D.F. from the "among" row in the GLM table as the numerator *df* and the D.F. from the "within" row as the denominator *df*. Enter the F value under the $F_{(0.05)}$ column in the final GLM table.

Example:

In the GLM table in step 2b, the numerator *df* is 2 and the denominator *df* is 27; therefore, the F value is 3.35.

d. Compute the least significant difference (LSD). These analyses are analogous to those used for the fathead minnow growth values (see SOP-12). The LSD is the minimum amount of fecundity that can be statistically determined from the data in the GLM table. The calculations are used to complete the following sentence in the final toxicity test report. "Asterisks in the summary table show concentrations significantly reducing survivorship of *Ceriodaphnia* according to Fisher's Exact Test and/or concentrations significantly lowering fecundity according to Dunnett's t-test (p = 0.05, one-tailed, t_d = _.__). The least significant difference in fecundity at the 5% level ($LSD_{0.05}$) is __.__ offspring per female, as computed from the error mean square term obtained by using the GLM analysis procedure."

Calculate the LSD using the following formula:

$$\text{LSD (in offspring per female)} = t_d \times \sqrt{\frac{2 \times \text{MSE}}{10}},$$

where t_d is Dunnett's t-value at 0.05 and MSE is the mean square error.

Dunnett's t (0.05) can be found in a statistical book (e.g., Steel and Torrie 1960, p. 446). Use alpha = 0.05. The "among" D.F. from the GLM table is p (the number of treatment means excluding the control), and the "within" D.F. from the GLM table is the error D.F. value along the left-hand side of the table for Dunnett's t-Test. Dunnett's t-value is found at the intersection of the p column and the error row. MSE can be found on the final GLM table: it is the mean square value in the "within" row.

Example:

In the GLM table in step 2b, the "among" D.F. value is 2 and the "within" D.F. value is 27; therefore, Dunnett's t (0.05) is 2.00. The MSE is 6.304. According to the LSD equation, the LSD is 2.25.

e. Determine whether or not the *Ceriodaphnia* in a particular concentration or site had significantly lower fecundity than the control. Subtract the LSD value from the value for mean reproduction of the controls. If any of the mean reproduction values for the concentrations are lower than this number, place an asterisk next to the reproduction number.

Example:

In the summary table in step 2b, the mean reproduction of the controls is 21.0. When the LSD (2.25) is subtracted from the mean reproduction of the controls (23.4), the remainder is 21.15. Because the mean reproduction value in the 10% concentration is less than 21.15, an asterisk is placed beside it.

f. Compute the LSD expressed as a percentage of the control. This information will be used to complete the following sentence in the final toxicity test report: "The LSD represents a __._% reduction in fecundity in relation to the control."

To calculate the LSD expressed as a percentage of the control, use the following equation.

$$\text{LSD as \% reduction} = 100 \times \frac{\text{LSD}}{\text{mean reproduction of controls}}.$$

Example:

In the GLM table in step 2a, the mean reproduction of the controls is 23.4. As calculated in step 2d, the LSD is 2.25. The LSD as a percentage of the control is therefore 9.6%.

SUBJECT: **THE FATHEAD MINNOW REFERENCE TEST**

Purpose

To establish the sensitivity of fathead minnow larvae maintained in the breeding cultures and used for toxicity tests.

Equipment

Isolated fathead minnows larvae (see SOP-9)
Cadmium chloride
Twenty-eight 100-mL glass beakers
Twenty-four 20-mL polystyrene beakers
Six 500-mL graduated cylinders
Six 100-mL graduated cylinders
Six 500-mL volumetric flasks
1.0-mL or 200-μL Pipetteman pipette
Mettler top-loading balance
Plexiglass racks for test vessels
Parafilm
Cadmium Hazardous Waste container
Binocular stereomicroscope
Magnifying lamp
Desktop magnifier or Wild microscope
Dilution and control water: one part mineral water to nine parts deionized distilled water (see SOP-1)
Registered fathead minnow log book

Procedure

1. Isolate larvae. Using a polished glass tube and pipette bulb, transfer six larvae that are <24 h old from the hatching chamber to a 20-mL polystyrene beaker containing 5-8 mL of control water. Repeat this procedure until you have 42 beakers with six larvae in each.

2. Using cadmium chloride (which is readily soluble) and deionized distilled water, prepare six stock solutions of cadmium in 500-mL volumetric flasks. The concentrations (CONC) of cadmium are as follows:

   A = 29.0 mg Cd/L,
   B = 22.6 mg Cd/L,
   C = 17.0 mg Cd/L,
   D = 14.0 mg Cd/L,
   E = 11.0 mg Cd/L, and
   F = 8.6 mg Cd/L.

   Label the flasks accordingly.

3. Make two sets of six polystyrene beakers, labeling (coding) the beakers in each set: A, B, C, D, E, and F. Label six 500-mL graduated cylinders: A, B, C, D, E, and F.

4. Pour a small amount of stock A (slightly more than 5 mL) into a polystyrene beaker labeled "A." Then recap volumetric flask A. Pour the same amount of stock B into a beaker labeled "B." Then recap volumetric flask B. Continue until this is done for stocks A through F.

5. Using the top-loading balance, tare beaker A from the second set of polystyrene beakers. Then, using a Pipetteman pipette, carefully weigh out 4.267 g of solution A from the beaker A, and put it in tared beaker A. Repeat this procedure for beakers B, C, D, E, and F.

6. Prepare cadmium solutions for the test as follows. Fill a 250-mL beaker with control water. Pour about 5-15 mL of control water from the 250-mL beaker into beaker A, which contains 4.267 g of stock A. Pour everything from beaker A into a graduated cylinder labeled "A"; do this at least three times. Bring the volume of solution in the graduated cylinder up to 320.0 mL with control water. Repeat this procedure for beakers B, C, D, E, and F, using corresponding stock solutions. Cover each cylinder with parafilm, and invert them several times to mix their contents.

7. Label clean 100-mL beakers for the replicates (REPs) in the test: A1, A2, A3, A4, B1, . . . F4. Fill the REP beakers with the corresponding test solution. Label four control (CONT) beakers: CONT 1, CONT 2, CONT 3, and CONT 4. Fill the CONT beakers with the control water.

8. Into each beaker, transfer six larvae from those isolated in step 1a.

9. In the registered fathead minnow log book, record the time you start adding animals to the test solution and the time when the last animal has been added.

10. Twenty-four hours after the test has been started, inspect the animals in each beaker. If necessary, use the Wild microscope or the desktop magnifier. In the registered fathead minnow log book, record the number of dead and live animals, using the data sheet in Exhibit SOP-14.1.

11. Dispose of all solutions containing cadmium by putting them in the container labeled "Cadmium Hazardous Waste."

SUBJECT: THE FATHEAD MINNOW REFERENCE TEST

DATA SHEET FOR FATHEAD MINNOW REFERENCE TEST

Date: _____ Test no.: _____ Initials: _____

Time test started: from _____ to _____

CONC (mg/L)	CODE	REP	Starting number	Number of dead at 24 h
0.386	A	1		
0.386	A	2		
0.386	A	3		
0.386	A	4		
0.300	B	1		
0.300	B	2		
0.300	B	3		
0.300	B	4		
0.226	C	1		
0.226	C	2		
0.226	C	3		
0.226	C	4		
0.186	D	1		
0.186	D	2		
0.186	D	3		
0.186	D	4		
0.146	E	1		
0.146	E	2		
0.146	E	3		
0.146	E	4		
0.114	F	1		
0.114	F	2		
0.114	F	3		
0.114	F	4		
0.00	CONT	1		
0.00	CONT	2		
0.00	CONT	3		
0.00	CONT	4		

Time test ended: from _____ to _____

PROBIT or MOVING AVERAGE LC_{50}: _____ mg Cd/L

95% C.L. = _____ - _____ mg Cd/L. Initials: _____

Control chart info: No. of standard tests completed: _____

Central tendency: _____ ± _____ mg Cd/L. Initials: _____

Exhibit SOP-14.1 Data sheet for fathead minnow reference test.

SUBJECT: **THE *CERIODAPHNIA* REFERENCE TEST**

Purpose

To establish the sensitivity of *Ceriodaphnia* maintained in the breeding cultures and used for toxicity tests.

Equipment

Isolated *Ceriodaphnia* (see SOP-10)
Cadmium chloride
Twenty-eight 100-mL glass beakers
Twenty-four 20-mL polystyrene beakers
Six 500-mL graduated cylinders
Six 100-mL graduated cylinders
Six 500-mL volumetric flasks
1.0-mL or 200-μL Pipetteman pipette
Mettler top-loading balance
Styrofoam racks for test vessels
Parafilm
Cadmium Hazardous Waste container
Binocular stereomicroscope
Magnifying lamp
Desktop magnifier or Wild microscope
Registered *Ceriodaphnia* log book
Dilution and control water: one part mineral water (e.g., Perrier) to nine parts deionized distilled water
Deionized distilled water

Procedure

1. Isolate neonates (see SOP-10, step 2a.).

2. Using cadmium chloride and distilled deionized water, prepare six stock solutions of cadmium in 500-mL volumetric flasks. The concentrations (CONC) are as follows:

   A = 40.0 mg Cd/L,
   B = 30.0 mg Cd/L,
   C = 20.0 mg Cd/L,
   D = 15.0 mg Cd/L,
   E = 10.0 mg Cd/L, and
   F = 5.0 mg Cd/L.

   Label the flasks accordingly.

3. Make two sets of six polystyrene beakers, labeling (coding) the beakers in each set: A, B, C, D, E, and F. Label six 100-mL graduated cylinders: A, B, C, D, E, and F.

4. Pour a small amount of stock A (slightly more than 5 mL) into a polystyrene beaker labeled "A." Then recap volumetric flask A. Pour the same amount of stock B into a beaker labeled "B." Then recap volumetric flask B. Continue until this until stocks A through F have been poured into beakers.

5. Using a Mettler top-loading balance, tare beaker A from the second set of polystyrene beakers. Then, using a Pipetteman pipette, carefully weigh out 1.000 g of solution A from beaker A, and put it in tared beaker A. Repeat this procedure for beakers B, C, D, E, and F.

6. Prepare cadmium solutions for the test as follows. Fill a 500-mL beaker with control water. Pour about 5-15 mL of control water from the 500-mL beaker into beaker A, which contains 1.000 g of stock A. Pour everything from beaker A into a graduated cylinder labeled "A"; do this at least three times. Bring the volume of solution in the graduated cylinder up to 75.0 mL with control water. Repeat this procedure for beakers B, C, D, E, and F. Cover each cylinder with parafilm and invert several times to mix.

7. Label clean polystyrene beakers for the replicates (REPs) in the test: A1, A2, A3, A4, B1, . . . F4. Label four control (CONT) beakers: CONT 1, CONT 2, CONT 3, and CONT 4. Fill the CONT beakers with 15-17 mL of control water. Fill the REP beakers with their corresponding test solution.

8. Into each beaker, transfer six neonates from those collected in step 1.

9. In the registered *Ceriodaphnia* log book, record the time you start adding animals to the test solution and the time when the last animal has been added.

10. Twenty-four hours after the test has been started, inspect the animals in each beaker. Use a microscope or desktop magnifier to determine if each animal is alive or dead. In the registered *Ceriodaphnia* log book, record the number dead and the number alive, using the data sheet in Exhibit SOP-15.1.

11. Dispose of all solutions containing cadmium by putting them in the container labeled "Cadmium Hazardous Waste."

SUBJECT: THE *CERIODAPHNIA* REFERENCE TEST

DATA SHEET FOR *CERIODAPHNIA* REFERENCE TEST

Date: _____ Test no.: CR-_____ Initials: _____

Time test started: from _____ to _____ Water batch: _____

CONC (mg/L)	CODE	REP	Starting number	Number of dead at 24 h
0.400	A	1		
0.400	A	2		
0.400	A	3		
0.400	A	4		
0.300	B	1		
0.300	B	2		
0.300	B	3		
0.300	B	4		
0.200	C	1		
0.200	C	2		
0.200	C	3		
0.200	C	4		
0.150	D	1		
0.150	D	2		
0.150	D	3		
0.150	D	4		
0.100	E	1		
0.100	E	2		
0.100	E	3		
0.100	E	4		
0.050	F	1		
0.050	F	2		
0.050	F	3		
0.050	F	4		
0.00	CONT	1		
0.00	CONT	2		
0.00	CONT	3		
0.00	CONT	4		

Time test ended: from _____ to _____

PROBIT or MOVING AVERAGE LC_{50}: _____ mg Cd/L

95% C.L. = _____ - _____ mg Cd/L. Initials: _____

Control chart info: No. of standard tests completed: _____

Central tendency: _____ ± _____ mg Cd/L. Initials: _____

Exhibit SOP-15.1 Data sheet for *Ceriodaphnia* reference test.

SUBJECT: **DATA LOGGING AND CARE OF TECHNICAL NOTEBOOKS**

Purpose

To maintain clear, legible research records.

Equipment

Registered technical notebooks from Laboratory Records in Building 4500-North.

Procedure

1. Keep good research records, using the proper registered technical notebook for each task.

2. Return notebooks to Laboratory Records upon the termination of the research records, upon termination of your employment, upon termination of the program or project, or at the time that the notebooks cease to be of reference value.

3. Write entries legibly with a ball point pen.

4. Date and sign each entry you make. Arrange entries in chronological order.

5. Make alterations or amplifications by adding new entries with the current date, and cross reference them to previous entries. DO NOT OBLITERATE OR BACK DATE ANY ENTRY. DO NOT USE CORRECTION FLUID.

6. Date and sign all data sheets and charts, as well as all drawings and graphs drawn on special paper, and securely fasten them in place, using glue or other means. Do not obscure any information.

7. Preface each new item (e.g., each fathead minnow toxicity test) with a brief description of the purpose and planned approach (e.g., water sample, concentrations tested, and concentration of stock solution).

8. For more information, see the following copy of *Technical Notebooks as Research Records.*

TECHNICAL NOTEBOOKS
AS
RESEARCH RECORDS

THEIR PURPOSE AND USE

Prepared by

PATENT SECTION
OFFICE OF GENERAL COUNSEL

MARTIN MARIETTA ENERGY SYSTEMS, INC.
POST OFFICE BOX Y
OAK RIDGE, TENNESSEE 37831

INTRODUCTION

Your technical notebook has been issued as a convenience for recording your ideas, experimental endeavors, and the data and results thereof. The pages are bound so that portions of the information cannot be misplaced or lost. The notebook has an identification number, and the pages are serially numbered for ease in locating and identifying the contents.

In using your notebook remember that its contents should serve many purposes, and the care you use in recording the information will affect its use. A well-kept notebook will provide a useful document which will: (1) avoid future duplication of efforts, (2) assist in the development of a fundamental approach to a solution of a technical problem, (3) serve as a source of information for reports and technical talks, and (4) provide records for patent purposes.

It is therefore necessary that entries in the notebook should answer these questions:

1. What was done?
2. When was it done?
3. Who did it?
4. Who knows about it?

By following the guides set forth below, these questions can be answered at any subsequent date – even after several years, when memory may fail.

WHAT WAS DONE

Entries should be made in ink whenever it is reasonable to do so, although pencil notations are acceptable. Remember, the records must be readable many years hence.

It is highly advisable to preface each new item, such as a process, machine design, composition of matter, method, etc., with a brief description of the purpose of the work so that you will not have to guess if asked to interpret the data several years later. It is also an aid to others who may be carrying on that particular line of investigation at a later date.

The data should be entered directly in the notebook, if possible. When this is impossible, records can be made on separate sheets of paper and transferred to the notebook the same day. In the event that some unavoidable interruption prevents entering the data the same day, it should be entered as soon as possible thereafter under the date of entry.

NEVER back-date on entry!

Charts, graphs, small drawings, etc., drawn on special paper should be glued or otherwise securely fastened in place. There should be some narrative reference to these supplemental pages in the notebook entries for explanation.

In some instances essential technical information will not be in a form convenient for entry in the notebook, as in the case of computer outputs, large drawings, tape recordings, etc. In these cases, a narrative summary should be made in the notebook and some identifying designation given to the evidence and referenced in the summary. In this way, the information may be located when required. Furthermore, the entry of the summary establishes a date for the supporting data.

Never erase or smudge out an entry or remove pages from the notebook. If an entry is wrong, draw a line through it and continue in a new space. It is not necessary to state why the data are in error, but it is a good plan to do so because the explanation is useful in refreshing your memory at a later date. Remember, the recording of negative results may help prevent duplication of effort.

WHEN WAS IT DONE

Every page or entry should bear a date. If several entries made the same day are on one page, a single date at the top of the page; is sufficient. Where entries on a page are not all made on the same day, each entry should bear a date even though there are several.

Ordinarily the notebook will contain entries in chronological order; therefore, any deviation from this order should be plainly marked and explained if possible. The previously mentioned charts, graphs, drawings, etc., should be dated like any other entry and placed in the notebook in as close to chronological order as possible.

WHO DID IT

Entries will normally be made by the individual to whom the notebook has been issued, and each will be signed by him. Entries by others should be signed by them.

Credit should be given to individuals who have made suggestions relating to the development so that in case an invention is made, there will be no problem of inventorship. Credit should also be given to anyone performing work relating to the development so that their contribution can be weighed in determing inventorship.

In cases where work is carried on in cooperation with other organizations, personal conferences are often necessary to discuss new developments; Such conferences should always be entered in your notebook. The entries should give the date of the conference, a list of those present, and an outline of the subjects discussed. This will nullify occasional claims of other parties that you have appropriated information received from them.

WHO KNOWS ABOUT IT

In order to get the notebook admitted as evidence in a controversy, you must establish its authenticity. This is done by your testimony and recognition of your own handwriting and your signature. In addition, your evidence must be corroborated by independent witnesses. In order that you may know whom to call at some future date, you should have your notebook reviewed by persons in your department. They should be familiar with your work but should not be working on the same problem with you. They should sign their names and the date at the end of a section of your notebook which they reviewed, indicating that they read and understood the foregoing entries. Witnessing stamps[1] are available for this purpose in most Department offices. It is suggested that your notebook be witnessed periodically; for example, once a week. Witnessing of supplemental pages is also essential.

In addition to the above-mentioned witnesses, it is well to mention any other persons who are familiar with the development and explain their association with it. As stated in the previous section, it is advisable to list any other organizations or individuals who have knowledge of the development. This includes organizations with whom you have had personal conferences or to whom you have sent drawings or other technical data.

[1]See sample entry on last page.

SUMMARY

The keeping of good records of research is an essential part of your professional responsibility. The quality of your technical reports, publications, etc., may well depend upon the care with which you record data in your technical notebook. Furthermore, the success and promptness with which you complete your research will be influenced by the notebook entries. Last, but not least, your records – in particular your technical notebook – may determine the outcome of patent proceedings to establish the first inventor.

For all these purposes, your notebook entries must provide information as to "what, when, and who." Make sure that your notebook complies.

FREQUENT OMISSIONS

1. Description of experiment.
2. Date of entries.
3. Original data.
4. Signature of experimentor.
5. Credit for ideas.
6. Signature of witnesses.
7. Official notebook number.

SUBJECT: **DATA LOGGING AND CARE OF TECHNICAL NOTEBOOKS**

SAMPLE ENTRY

75

DATE June 14, 1984

DESCRIBE to dissolve uranium as to extract it in solvents having supply of reagents arrive.
Herman L. Dooks

THE FOREGOING PAGES NUMBERED 67-75
DESCRIBING URANIUM RECOVERY
READ AND UNDERSTOOD BY ME, THIS 15th DAY OF June, 19 84

WITNESS John Public
Jane Doe

PATENT SECTION OFFICES

PRODUCTION PLANTS

K-25 Telephone 6-0349
Y-12 Telephone 4-2229

OAK RIDGE NATIONAL LABORATORY

Telephone 4-4177

SUBJECT: **REFERENCE PROCEDURES--CONTENTS**

This section of the manual cites the specific national testing procedures, or reference procedures, upon which the standard operating procedures of the ESD Toxicology Laboratory are based.

REF-1: HEALTH AND SAFETY

REF-2: QUALITY ASSURANCE

REF-3: FACILITIES AND EQUIPMENT

REF-4: DILUTION WATER

REF-5: EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

REF-6: CHRONIC TOXICITY TEST END POINTS AND DATA ANALYSIS

REF-7: FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

REF-8: RECOMMENDED DEFINITIVE TEST CONDITIONS FOR FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS

REF-9: *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

REF-10: RECOMMENDED DEFINITIVE TEST CONDITIONS FOR *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST USED FOR NPDES PERMITS

SUBJECT: HEALTH AND SAFETY

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 6-7.

SECTION 3

HEALTH AND SAFETY[1]

3.1 GENERAL PRECAUTIONS

3.1.1 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.2 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.2 SAFETY EQUIPMENT

3.2.1 Personal Safety Gear

Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 Laboratory Safety Equipment

Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1. Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals, Paragraph 3.5). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.3.2. Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized.

3.3.3. It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4. All containers should be adequately labeled to indicate their contents.

[1]Adapted from: Peltier and Weber (1985).

3.3.5. Good housekeeping contributes to safety and reliable results.

3.3.6. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.7. Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, and polio.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals, including USEPA (1977), and Walters and Jameson (1984).

SUBJECT: **QUALITY ASSURANCE**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W.B. Horning and C.I. Weber, 1985, pp. 8-12.

SECTION 4

QUALITY ASSURANCE[1]

4.1 INTRODUCTION

4.1.1 Quality Assurance (QA) practices for effluent toxicity tests consist of all aspects of the test that affect data quality, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation. For general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1979d, 1980b, and 1980c; and DeWoskin, 1984.

4.2 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.2.1 Effluent samples collected for on-site and off-site testing must be preserved as described in Section 8, Effluent and Receiving Water Sampling and Sample Handling.

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, Pimephales promelas, the cladoceran, Ceriodaphnia dubia, and the green alga, Selenastrum capricornutum. The organisms used should be disease-free, and should be positively identified to species.

4.4 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.4.1 Laboratory and bioassay temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities and Equipment).

4.5 ANALYTICAL METHODS

4.5.1 Routine chemical and physical analyses must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a,b).

4.6 CALIBRATION AND STANDARDIZATION

4.6.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, conductivity, alkalinity, and hardness, must be calibrated and standardized according to instrument manufacturers procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1, USEPA, 1979b).

[1]Adapted from: Peltier (1978), Peltier and Weber (1985), and USEPA (1979a).

4.6.2 Wet chemical methods used to measure hardness and alkalinity must be standardized according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1, USEPA 1979b).

4.7 DILUTION WATER

4.7.1 The dilution water used in the toxicity tests may be synthetic water, receiving water, or ground water, appropriate to the objectives of the study and logistical constraints, as discussed in Section 7.

4.8 TEST CONDITIONS

4.8.1 Water temperature must be maintained within the limits specified for each test. Dissolved oxygen (DO) concentrations and pH should be checked at the beginning of the test and daily throughout the test period.

4.9 TEST ACCEPTABILITY

4.9.1 The results of the fathead minnow or Ceriodaphnia 24-h reference toxicant tests are unacceptable if the survival in the controls is less than 90%. For effluent toxicity tests to be acceptable, control survival must be at least 80%. The results of the algal toxicity test are unacceptable if the cell density in the controls after 96 h is less than 10^6 cells/mL.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the best professional judgment and experience of the investigator. The deviation from test specifications must be noted when reporting data from the test.

4.10 PRECISION

4.10.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single laboratory precision of each type of test to be used in a laboratory should be determined by performing five or more tests with a reference toxicant. In cases where the test data are used in the Probit Analysis (see Section 9), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated end points from the replicated tests. However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9), precision can only be described by listing the NOEC-LOEC interval for each test. In this case, it is not possible to express precision in terms of a commonly used statistic. For instance, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. However, the "true" no effect concentration could fall anywhere within the interval, NOEC $\pm$ (NOEC-LOEC).

4.10.2 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. Other factors which can affect test precision include test organism age, condition, and sensitivity, temperature control, and feeding.

4.11 REPLICATION AND TEST SENSITIVITY

4.11.1 The sensitivity of the tests will depend in part on the number of replicates, the probability level selected, and the type of statistical analysis. The minimum recommended number of replicates varies with the test and the statistical method used, and is discussed in Section 9 and in each method. The sensitivity of the test will increase as the number of replicates is increased.

4.12 QUALITY OF TEST ORGANISMS

4.12.1 If the laboratory does not have an ongoing test organism culturing program and obtains the test organisms from an outside source, the sensitivity of each batch of test organisms must be evaluated with a reference toxicant in a toxicity test performed concurrently with the effluent and/or receiving water toxicity tests. If the laboratory maintains breeding cultures, the sensitivity of the offspring should be determined in a toxicity test performed with a reference toxicant at least once each month. If preferred, this reference toxicant test may be performed concurrently with an effluent toxicity test. A 24-h acute toxicity test is used to determine the sensitivity of fathead minnows and Ceriodaphnia. For the acute toxicity test design, see Peltier and Weber (1985). A 96-h toxicity test is used to determine the sensitivity of Selenastrum.

4.12.2 The sensitivity of fathead minnow larvae is determined with newly hatched larvae as used in the growth and survival or embryo-larval test. The sensitivity of Ceriodaphnia is determined using animals less than 24 h old, and which were released within the same 4-h period.

4.12.3 Three reference toxicants are available from EMSL-Cincinnati to establish the precision and validity of toxicity data generated by biomonitoring laboratories: sodium dodecylsulfate (SDS), sodium pentachlorophenate (NaPCP), and cadmium chloride ($CdCl_2$). The reference toxicants may be obtained by contacting the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268; FTS 684-7325, comm'l 513-569-7325. Instructions for the use and the expected toxicity values for the reference toxicants are provided with the samples. To assure comparability of QA

data on a national scale, all laboratories must use the same source of reference toxicant (EMSL-Cincinnati) and the same formulation of dilution water -- moderately hard synthetic water, described in Table 1, p. 18, for fathead minnows and Ceriodaphnia, and algal growth medium described in Tables 1 and 2, Section 14, for Selenastrum.

4.13 FOOD QUALITY

4.13.1 The quality of the food for fish and invertebrates is an important factor in toxicity tests. Suitable trout chow, Artemia, and other foods must be obtained as described in the manual. Limited quantities of reference Artemia cysts, information on commerical sources of good quality Artemia cysts, and procedures for determining cyst suitability as food are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268. The suitability of each new supply of food must be determined in a side-by-side test in which the response of test organisms fed with the new food is compared with the response of organisms fed a reference food or a previously used, satisfactory food.

4.14 CONTROL CHARTS

4.14.1 A control chart should be prepared for each reference-toxicant-organism combination, and successive toxicity values should be plotted and examined to determine if the results are within prescribed limits (Fig. 1). In this technique, a running plot is maintained for the toxicity values (X_i) from successive tests with a given reference toxicant. The type of control chart illustrated (USEPA, 1979a) is used to evaluate the cumulative trend of the statistics from a series of samples. The mean (X) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive point, until the statistics stabilize. Outliers, which are values which fall outside the upper and lower control limits, and trends of increasing or decreasing sensitivity are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone.

4.14.2 If the toxicity value from a given test with the reference toxicant does not fall in the expected range for the test organisms when using the standard dilution water, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

4.15 RECORD KEEPING

4.15.1 Proper record keeping is required. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt, and other pertinent information relating to their history and health, and information on the calibration of equipment and instruments, test conditions employed, and test results. Annotations should be made on a real-time basis to prevent the loss of information.

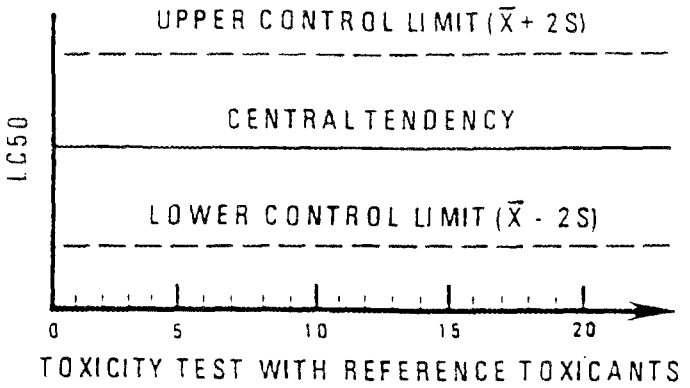

Figure 1. Control chart.

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 / n}{n-1}}$$

Where:

X_i = Successive LC50's from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean LC50.

S = Standard deviation.

SUBJECT: **FACILITIES AND EQUIPMENT**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 13-14.

SECTION 5

FACILITIES AND EQUIPMENT[1]

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing, holding, and acclimating organisms. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, surface water, dechlorinated tap water, or synthetic water. Dechlorination can be accomplished by aeration (allowing the water to stand in an open vessel for 24 h), carbon filtration, or the use of sodium thiosulfate. Use of 1.0 mg (anhydrous) sodium thiosulfate/L will reduce 1.5 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and fumes. Test facilities must be well ventilated and free of fumes. During rearing, holding, acclimating, and testing, test organisms should be shielded from external disturbances.

5.1.2 Materials used for exposure chambers, tubing, etc., which come in contact with the effluent should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON^R) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Plastics such as polyethylene, polypropylene, polyvinyl chloride, TYGON^R, etc., may be used as test chambers or to store effluents, but caution should be exercised in their use because they could introduce toxicants when new, or carry over toxicants from one test to another if reused. The use of glass carboys is discouraged for safety reasons.

5.1.3 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with the test media before using in the test. Copper, galvanized material, rubber, brass, and lead must not come in contact with holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

[1]Adapted from: Peltier and Weber (1985).

5.1.4 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each test.

5.3 CLEANING

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require rigorous cleaning. It is sufficient to rinse the new containers once with sample before use. New glassware, however, should be soaked overnight in acid (see below).

5.3.2 It is recommended that all sample containers, test vessels, pumps, tanks, and other equipment that has come in contact with effluent be washed after use in the manner described below to remove surface contaminants. Special cleaning requirements for glassware used in algal toxicity tests are described in Section 14.

1. Soak 15 minutes, and scrub with detergent in tap water, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh dilute (20% V:V) nitric acid or hydrochloric acid to remove scale, metals and bases. To prepare a 20% solution of acid, add 20 mL of concentrated acid to 80 mL of distilled water.
4. Rinse twice with tap water.
5. Rinse once with full-strength acetone to remove organic compounds.
6. Rinse well with tap water.
7. Rince twice with dilution water.

5.3.3 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

SUBJECT: **DILUTION WATER**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 17-18.

SECTION 7

DILUTION WATER

7.1 The source of dilution water used in the tests will depend largely on the objectives of the study as described in Section 1: (1) If the objective of the test is to estimate the inherent chronic toxicity of the effluent, a standard dilution water (moderately hard water, Table 1) is used; (2) If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of non-toxic receiving water collected upstream from the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same hardness (± 10%) as the receiving water; (3) If the objective of the test is to determine the additive effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected daily upstream from the outfall.

7.2 When the dilution water is to be taken from the receiving water "upstream" from the outfall, it should be collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent. The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature until used.

7.3 Where toxicity-free dilution water is required in a test, the water is considered acceptable if test organisms show adequate survival, growth, and reproduction in the controls during the test.

7.4 Dechlorinated water should be used as dilution water only as a last resort, because it is usually difficult to completely remove all the residual chlorine or chlorinated organics, which may be very toxic to the test organisms. Sodium thiosulfate is recommended for dechlorination (1.0 mg anhydrous sodium thiosulfate/L will reduce 1.5 mg chlorine/L). After dechlorination, total residual chlorine must be non-detectable.

7.5 If it is necessary to pass the dilution water through a deionizer to remove unacceptably high concentrations of copper, lead, zinc, fluoride, or other toxic substances before use, it must be reconstituted to restore the calcium and magnesium removed by the deionization process.

7.6 To prepare a synthetic fresh water, use the reagents listed in Table 1. For example, to prepare 20 L of moderately hard synthetic water:

1. Place 19 L of distilled or deionized water in a properly cleaned plastic carboy.
2. Add sufficient $MgSO_4$, $NaHCO_3$ and KCl to the carboy, and stir well.

3. Add sufficient $CaSO_4.2H_2O$ to 1 L of distilled or deionized water in a separate flask, place on a magnetic stirrer until the calcium sulfate has dissolved and add to the carboy and stir well.
4. Aerate vigorously for 24 h (with air filtered through cotton to remove oil) to dissolve the added chemicals and stabilize the medium.

7.7 The measured pH, hardness and alkalinity of the aerated water will be approximately as indicated under "Final Water Quality" in Table 1.

TABLE 1. PREPARATION OF SYNTHETIC FRESH WATER[a]

	Reagent Added (mg/L)[b]				Final Water Quality		
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCL	pH[c]	Hardness[d]	Alkalinity[d]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[a]Taken in part from Marking and Dawson (1973).
[b]Add reagent grade chemicals to distilled or deionized water.
[c]Approximate equilibrium pH after 24 h of aeration.
[d]Expressed as mg $CaCO_3$/L .

SUBJECT: **EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 19-21.

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point usually should be the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and averages the quality of the effluent over the sampling period. A lengthy discussion of the advantages and disadvantages of using grab or composite samples is found in Peltier and Weber (1985).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Definitive tests performed for NPDES permit purposes require <u>daily</u> effluent sample collection and daily renewal of test solutions.

8.2 RECEIVING WATER SAMPLING

8.2.1 It is common practice to collect grab samples for receiving water toxicity studies.

8.2.2 When non-toxic receiving water is required for a test, it may be collected upstream from the outfall or from other uncontaminated surface water having approximately the same hardness (± 10%) as the receiving water. If the objective of the test is to determine the additive effects of the discharge on receiving water which may already be contaminated, the test is performed using dilution water consisting of receiving water collected daily upstream from the outfall.

8.2.3 Dilution water to be taken from the receiving water "upstream" from the outfall, is collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent.

8.2.4 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall may be difficult to ascertain, and it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.3 SAMPLE HANDLING AND PRESERVATION

8.3.1 If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time from collection of a grab or composite sample and the initiation of the test must not exceed 72 h. Composite samples should be chilled during collection, where possible. Except when used within 24 h of collection, samples must be chilled after collection and maintained at 4°C until used.

8.3.2 Samples Used in On-Site Tests

8.3.2.1 Samples collected for on-site tests should be used within 24 h.

8.3.3 Samples Shipped to Off-Site Facilities

8.3.3.1 Samples collected for off-site toxicity testing are to be chilled to 4°C when collected, shipped iced to the central laboratory, and there transferred to a refrigerator (4°C) until used. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory.

8.3.3.2 Samples may be shipped in 4-L (1-gal) glass jugs, CUBITAINERS^R, or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. Glass jugs can be cleaned and reused (see p. 12), whereas CUBITAINERS^R and plastic jugs are not reused. Plastic containers used for effluents or toxic surface water samples should be punctured after use to prevent reuse.

8.4 SAMPLE PREPARATION

8.4.1 With the Ceriodaphnia and fathead minnow tests, effluents and surface waters must be filtered through a (30 um) plankton net to remove indigenous organims that may attack or be confused with the test organisms (see Ceriodaphnia test method for details). Surface waters used in algal

toxicity tests must be filtered through a 0.45 um pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm holes to remove debris and/or break up large floating or suspended solids.

8.4.2 The DO concentration in the dilution water should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH.

8.4.3 If the dilution water and effluent must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved gases may become a problem. To prevent this problem, the effluent and dilution water are heated to 25°C and checked for dissolved oxygen (DO) with a probe. If the DO exceeds 8.5 mg/L (100% saturation), the solutions are aerated virorously with an air stone (usually 1-2 min) until the DO is lowered to 100% saturation.

SUBJECT: **CHRONIC TOXICITY TEST END POINTS AND DATA ANALYSIS**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 22-25.

SECTION 9

CHRONIC TOXICITY TEST END POINTS AND DATA ANALYSIS

9.1 END POINTS

9.1.1 Numerous terms are used to define the end points employed in chronic toxicity tests, which have their origin in the earlier, full life-cycle tests. As shorter "chronic" tests were developed, it became common practice to apply the same terminology to the end points. The primary terms in current use are listed below:

9.1.1.1 Safe Concentration - the highest concentration which will permit normal propagation of fish and other aquatic life in receiving waters.

9.1.1.2 No Observed Effect Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle test, which causes no statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and reproduction).

9.1.1.2. Lowest Observed Effect Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle test, which causes a statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and reproduction).

9.1.1.3. Maximum Acceptable Toxicant Concentration (MATC) - An undetermined concentration within the interval bounded by the NOEC and LOEC.

9.1.1.4. Chronic Value (ChV) - A value lying between the NOEC and LOEC; derived by calculating the geometric mean of the NOEC and LOEC. The term is sometimes used interchangably with MATC.

9.1.1.5. LC (or EC) - Lethal concentration (LC) or effective concentration (EC). A point estimate of the toxicant concentration that would adversely affect a given percent of the test organisms, calculated by regression (such as Probit Analysis). The LC1 (or EC1) is the estimated concentration of toxicant that adversely affects 1% of the test population, and is defined here as the threshold concentration, or lowest concentration that would cause an adverse effect on the observed parameters, and falls in the range of the NOEC and LOEC (Birge, et. al. 1981).

9.2 DATA ANALYSIS

9.2.1 Role of the Statistician

9.2.1.1 The choice of a statistical method to analyze toxicity test data and the interpretation of the results of the analysis of the data from any

of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.2.2 Plotting the Data

9.2.2.1 It is recommended that the data always be plotted as a preliminary step, to help spot problems and detect unsuspected trends or patterns in the responses.

9.2.3 Data Transformations

9.2.3.1 Transformations of the data, such as arc sine and logs, can be used if they help the data meet the assumptions of the proposed analyses.

9.2.4 Analysis of Growth and Reproduction Data

9.2.4.1 Growth data from the fathead minnow larval survival and growth test, and reproduction data from the Ceriodaphnia survival and reproduction test, are analyzed using Dunnett's Procedure (Dunnett, 1955) if the assumptions of normality and homogeneity of variance are met (see Appendix for details). If the assumptions are not met, the data are analyzed using Steel's Many-One Rank Test (Steel, 1959; Miller, 1981).

9.2.4.2 The growth response data from the algal toxicity test may be converted to a proportion of the growth of the controls, which may then be analyzed by Probit Analysis (Finney, 1971) or, the growth response data, after an appropriate transformation if necessary to meet the assumptions of normality and homogeneity of variance, may be analyzed by Dunnett's Procedure or Steel's Many-One Rank Test.

9.2.5 Analysis of Mortality Data

9.2.5.1 Mortality data from the fathead minnow larval survival and growth test and the fathead minnow embryo-larval survival and teratogenicity test are used in a Probit Analysis to determine the LC1, if Probit Analysis is appropriate (see discussion below).

9.2.5.2 Fisher's Exact Test is used to analyze the mortality data from the Ceriodaphnia survival and reproduction test, prior to the analysis of the reproduction data.

9.2.5.3 Mortality data from the fathead minnow larval survival and growth test, and the fathead minnow embryo-larval survival and teratogenicity test, can be analyzed by Dunnett's Procedure or Steel's Many-One Rank Test after transforming the square root of the proportion of dead organisms to an arc sine value (see Appendix). This transformation is performed by the computer program for Dunnett's Procedure provided in the Appendix.

9.2.6 Dunnett's Procedure

9.2.6.1 Dunnett's Procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison method for comparing each of the treatment means with the control mean, in a series of paired tests. Use of Dunnett's Procedure requires at least two replicates per treatment.

9.2.6.2 The asumptions upon which the use of Dunnett's Procedure are contingent are that the observations are independent and normally distributed, with homogeneity of variance. Before analyzing the data, the assumptions are checked using the procedures provided in the Appendix.

9.2.6.3 Some indication of the sensitivity of the analysis should be provided by calculating: (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test. Calculation of beta levels (Type II error, which results when the null hypothesis is not rejected when it should be) as an indication of the power of the test would be another alternative.

9.2.6.4 The safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of Dunnett's Procedure is provided in the Appendix.

9.2.6.5 If, after suitable transformations have been carried out, the normality assumptions have not been met, the Steel Many-One Rank Test should be used.

9.2.7 Steel's Many-One Rank Test

9.2.7.1 Steel's Many-One Rank Test is a multiple comparison method for comparing several treatments with a control which is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption for normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-One Rank Test can be much more efficient (Hodges and Lehmann, 1956). It is necessary to have at least four replicates to use Steel's test. The sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean.

9.2.7.2 The safe concentration is reported as the NOEC. A step-by-step example of Steel's Many-One Rank Test is provided in the Appendix.

9.2.8 Probit Analysis

9.2.8.1 Probit Analysis is used to analyze percentage data from concentration-response tests. The analysis can provide an estimate of the

concentration of toxicant lethal to a given percent of the test organisms and provide a confidence interval for the estimate. Probit Analysis also assumes normal distribution of log tolerances and independence of the individual responses. To use Probit Analysis, at least two partial mortalities must be obtained.

9.2.9 Fisher's Exact Test

9.2.9.1 Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test that the probability of a response is the same in two binomial populations. When used with the Ceriodaphnia data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a binomial population.

SUBJECT: **FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0**

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 28-41.

SUBJECT: **FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0**

SECTION 11

TEST METHOD[1,2]

FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

1. SCOPE AND APPLICATION

1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow (Pimephales promelas), using newly hatched larvae in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 Larvae (preferrably less than 24-h old) are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and growth (increase in weight) of the larvae.

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

[1]The format used for this method was taken from Kopp, 1983.
[2]This method was adapted from Norberg and Mount, 1985.

4.2 Adverse effects of low dissolved oxygen (DO) concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

4.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4.5 Food added during the test may sequester metals and other toxic substances and confound test results.

5. SAFETY

5.1 See Section 3, Health and Safety.

6. APPARATUS AND EQUIPMENT

6.1 Fathead minnow and brine shrimp culture units -- see Peltier and Weber (1985). This test requires 150-300 newly hatched larvae. It is preferable to obtain this fish from an inhouse fathead minnow culture unit. If it is not feasible to culture fish inhouse, newly hatched larvae can be shipped in well oxygenated water in insulated containers.

6.2 Samplers -- automatic sampler, preferrably with sample cooling capability, that can collect a 24-h composite sample of 4 L.

6.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

6.4 Environmental chamber or equivalent facility with temperature control (25± 2°C).

6.5 Water purification system -- Millipore Super-Q or equivalent.

6.6 Balance -- analytical, capable of accurately weighing larvae to 0.0001 g.

6.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

6.8 Test chambers -- borosilicate glass or non-toxic disposable plastic labware. A minimum of two 1-L beakers are required for each concentration and control. To avoid potential contamination from the air, the chambers should be covered during the test.

6.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

6.10 Volumetric pipets-- Class A, 1-100 mL.

6.11 Serological pipets-- 1-10 mL, graduated.

6.12 Pipet bulbs and fillers -- Propipet[R], or equivalent.

6.13 Droppers, and glass tubing with fire polished edges, 4mm ID -- for transferring larvae.

6.14 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

6.15 Glass or electronic thermometers -- for measuring water temperatures.

6.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1, USEPA 1979b).

6.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.19 Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section 5, Facilities and Equipment.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as activated-carbon-filtered distilled or deionized water that does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.5 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling.

7.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7.6 Laboratory quality assurance samples and standards for the above methods.

7.7 Specific conductivity standards (see USEPA Method 120.1, USEPA 1979b).

7.8 Reference toxicant solutions (see Section 4, Quality Assurance).

7.9 Formalin (4%) for use as a preservative for the fish larvae.

7.10 Brine Shrimp (Artemia) Cysts -- see Peltier and Weber (1985).

7.10.1 Although there are many commercial sources of brine shrimp eggs, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues. (One source is Aquarium Products, 180 L Penrod Ct., Glen Burnie, MD, 21061). Each new batch of Artemia cysts should be evaluated for nutritional suitability against known suitable reference cysts by performing a larval growth test. It is recommended that a sample of newly-hatched Artemia nauplii from each new batch of cysts be chemically analyzed to determine that the concentration of total organic chlorine does not exceed 0.15 ug/g wet weight or the total concentration of organochlorine pesticides plus PCBs does not exceed 0.3 ug/g wet weight (USEPA, 1982). If those values are exceeded, the Artemia should not be used.

7.10.2 Limited quantities of reference Artemia cysts, information on commerical sources of good quality Artemia cysts, and procedures for determining cyst suitability are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268.

7.11 Test organisms -- Newly-hatched fathead minnow larvae (see Peltier and Weber, 1985).

8. SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used, beginning at 10%. If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for daily renewal of two replicates per concentration, each containing 500 mL of test solution, is approximately 2 L. Prepare enough test solution (approximately 1400 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

11.2 START OF THE TEST

11.2.1 Tests should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to (25 ± 2°C) and maintained at that temperature until portions are added to the dilution water.

11.2.2 The test is initiated by placing larvae one or two at a time, into each test chamber in sequential order, until each chamber contains 10 larvae, for a total of at least 20 larvae for each concentration. The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.2.3 Randomize the position of test chambers at the beginning of the test.

11.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $uE/m^2/s$, or 50 to 100 foot

candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 2°C.

11.4 DISSOLVED OXYGEN (DO)

11.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should not fall below 40% saturation. If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, Kimax serological pipet, No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.5 FEEDING

11.5.1 The fish in each test chamber are fed 0.1 mL (approximately 700 to 1000) of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals (at the beginning, midway, and at the end of the work day). The nauplii should be rinsed with freshwater before use.

11.6 DAILY CLEANING OF TEST CHAMBERS

11.6.1 Before the daily renewal of test solutions, uneaten and dead brine shrimp and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL) fitted with a rubber bulb can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the larvae caught up in the siphon can be retrieved and returned to the chambers.

11.7 TEST SOLUTION RENEWAL

11.7.1 The test solutions are renewed daily using freshly collected samples, immediately after cleaning the test chambers. The water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (500 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence for the larvae.

11.8 ROUTINE CHEMICAL AND PHYSICAL ANALYSIS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO is measured at the beginning and end of each 24-h exposure period at all test concentrations and in the control.

11.8.1.2 Temperature, pH, and conductivity are measured at the beginning of each 24-h exposure period at all test concentrations and in the control.

11.8.1.3 Alkalinity and hardness are measured at the beginning of each 24-h exposure period in 100% effluent and in the control.

11.9 OBSERVATIONS DURING THE TEST

11.9.1 The number of live and dead larvae in each test chamber are recorded daily, and the dead larvae are discarded.

11.9.2 Protect the larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed during the performance of the above operations.

11.10 TERMINATION OF THE TEST

11.10.1 The test is terminated after seven days of exposure. At termination, the larvae in each test chamber are counted and preserved as a group, in 4% formalin, and are dried and weighed at a later date. Immediately prior to the dry weight analysis, the preserved larvae are rinsed in distilled water. The group of rinsed larvae from each test chamber are transferred to a tared weighing boat and dried at 100°C for a minimum of 2 h. Immediately upon removal from the drying oven, the weighing boats are placed in a dessicator to prevent the absorption of moisture from the air, until weighed. The weights should be measured to the nearest 0.1 mg.

11.11 ACCEPTABILITY OF TEST RESULTS

11.11.1 For the test results to be acceptable, survival in the controls must be at least 80%, except where survival in any test concentration is 80% or better.

11.12 SUMMARY OF TEST CONDITIONS

11.12.1 A summary of test conditions is listed in Table 1.

12. CALCULATIONS

12.1 The endpoints of toxicity tests using the fathead minnow larvae are based on the adverse effects on survival and growth. Probit Analysis (Finney, 1971), Dunnett's Procedure (Dunnett, 1955), and Steel's Many-One Rank Test (Steel, 1959; Miller, 1981), are used to evaluate the data. See the Appendix for examples of the manual computations, and the program listings and examples of data input and program output.

12.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in the Appendix. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.3 Table 2 shows the survival and growth response after seven days of exposure to the reference toxicant NaPCP. The data for an effluent would be similar except that the test concentrations would be expressed as a percent.

12.4 Analysis of Survival Data - Probit Analysis

12.4.1 Probit Analysis (Finney, 1971) is used to determine the concentration causing 1% mortality (LC1). In this analysis, the total survival data from all test replicates at a given concentration are combined (see Table 2, Mean Proportion Surviving). A listing of the computer program and an example of data input and program output for the Probit Analysis are provided in the Appendix. Note that for the data in Table 2, the number of animals exposed in each concentration would be 40, and number that died at each concentration could be calculated from the proportions in the table. The program transforms the concentration values to $\log_{10}$ and the percent mortality to probits, and then performs a regression analysis.

12.4.2 Report the LC1 and its 95% confidence limits. The LC1 is an estimate of the threshold (chronically toxic) concentration. For the sample data in Table 2, the LC1 is 128 ug NaPCP/L, with upper and lower 95% confidence intervals of 30.5 ug/L and 198 ug/L, respectively.

12.4.3 If the data do not meet the assumptions necessary for the Probit analysis, analyze the data using Dunnett's Test, as described below.

12.5 Analysis of Survival Data - Dunnett's Procedure

12.5.1 The survival data meet the normality assumptions (see Appendix), which indicates that analysis by Dunnett's Procedure is appropriate. If the data had not met the assumptions, Steel's Many-One Rank Test would have been used (see Section 9). Dunnett's Procedure (Dunnett, 1955; Steel and Torrie, 1960) includes an analysis of variance (ANOVA), followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means.

12.5.2 It is necessary to have at least duplicate test chambers at each treatment concentration to perform this test.

12.5.3 The computer program listed in the Appendix generates output which includes an ANOVA table, a statement about each treatment mean that can be used to identity the NOEC and LOEC, and the minimum difference between treatment and control means that can be detected as statistically significant.

12.5.4 The computer program makes the necessary transformation of the survival data by converting the square root of the proportion of surviving organisms to arc sine during the analysis, and includes a special modification of the arc sine transformation which is required where the

proportion of surviving organisms is 0 or 1 (Bartlett, 1937). For a more detailed information on the arc sine transformation, see the Appendix.

12.5.5 The results of the analysis of variance of the data from Table 2 are shown in Table 3, and indicate a statistically significant difference in survival among NaPCP concentrations.

12.5.6 The results of the comparison of the control with the treatment effects, using 18 degrees of freedom and with a Dunnett's "t" value of 2.41 (P = 0.05), indicate that the NOEC is Concentration 5 (256 ug/L) and the LOEC is Concentration 6 (512 ug/L). The computer printout of these results is as follows:

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2 (32 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3 (64 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4 (128 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5 (256 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6 (512 UG NAPCP/L) AND CONTROL.

12.5.7 For this set of data, the minimum difference that can be detected as statistically significant is 0.177. This represents a 19% reduction in the mean response (survival) from the control.

12.6 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

Log_{10} NOEC = Log_{10} 256 = 2.4082
Log_{10} LOEC = Log_{10} 512 = 2.7093
ChV = Antilog (2.4082 + 2.7093)/2 = Antilog 2.5588
ChV = 362 ug/L NaPCP

12.7 Analysis of Growth Data - Dunnett's Procedure

12.7.1 The use of Dunnett's Procedure to analyze the growth data, expressed in terms of average dry weight (Table 2), is similar to that for the survival data, except that the weight data are not transformed.

12.7.2. The average dry weight of the larvae from each replicate test chamber (Table 2) is entered into the program.

12.7.3. The results from the analysis of variance of the data in Table 2 are found in Table 4. The analysis indicates a statistically significant difference in the effects on larval growth at the various concentrations of NaPCP.

12.7.4. The results of the comparison of the average weights for each treatment with the average weights for the control, using 18 degrees of freedom and with a Dunnett's "t" value of 2.41 (P = 0.05), indicate that the NOEC is Concentration 4 (128 ug NaPCP/L) and the LOEC is Concentration 5 (256 ug NaPCP/L). The computer output from the Dunnett program is as follows (the control is Concentration 1):

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2 (32 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3 (64 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4 (128 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5 (256 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6 (512 UG NAPCP/L) AND CONTROL.

For this set of data, the minimum difference that can be detected as statistically significant is 0.095. This represents a 13.3% reduction in the mean response from the control.

12.6.5 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

Log_{10} NOEC = Log_{10} 128 = 2.1072
Log_{10} LOEC = Log_{10} 256 = 2.4082
ChV = Antilog (2.1072 + 2.4082)/2 = Antilog 2.2577
ChV = 181 ug/L NaPCP

12.6.6 The results of the test indicate that growth was a more sensitive index of the effects of NaPCP than was survival.

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Information on the single laboratory precision of the fathead minnow larval survival and growth test is presented in Table 5.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests can not be determined.

SUBJECT: **FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0**

TABLE 1. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 2°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 uE/m^2/s (50-100 ft-c)(ambient lab levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	1-L containers
7. Test solution volume:	500 mL/replicate
8. Renewal of test concentrations:	Daily
9. Age of test organisms:	Newly hatched larvaed
10. Larvae/test chamber and control:	10 larvae/chamber; Minimum of 20 larvae/test concentration
11. Replicate chambers/concentration:	Minimum of 2
12. Feeding regime:	Feed 0.1 mL newly hatched brine shrimp nauplii three times daily, 4 h between feedings (at the begining, midway, and the end of the work day)
13. Cleaning:	Siphon daily, immediately before test solution renewal
14. Aeration:	None, unless DO concentration falls below 40% saturation. Rate should be less than 100 bubbles/min.
15. Dilution water:	Moderately hard standard water, receiving water, other surface water, ground water, or synthetic water similar to receiving water
16. Effluent concentrations:	At least 5 and a control
17. Dilution factor:	Approximately 0.3 or 0.5
18. Test duration:	7 days
19. Effects measured:	Survival and growth (increase in weight)

TABLE 2. SUMMARY OF SURVIVAL AND GROWTH DATA OF FATHEAD MINNOW LARVAE EXPOSED FOR SEVEN DAYS TO SODIUM PENTACHLOROPHENATE

Test Conc. No.	NaPCP Conc. (ug/L)	Proportion of Survival in Replicate Chambers A	B	C	D	Mean Prop. Surv.	CV[a] (%)	Ave Dry Wgt (mg) In Replicate Chambers A	B	C	D	Mean Dry Wgt (mg)	CV (%)
1	Control	1.0	1.0	0.9	0.9	0.95	6	0.711	0.662	0.718	0.767	0.714	6
2	32	0.8	0.8	1.0	0.8	0.85	12	0.646	0.626	0.723	0.700	0.674	7
3	64	0.9	1.0	1.0	1.0	0.975	5	0.669	0.669	0.694	0.676	0.677	2
4	128	0.9	0.9	0.8	1.0	0.90	9	0.629	0.680	0.513	0.672	0.624	12
5	256	0.7	0.9	1.0	0.5	0.775	29	0.650	0.558	0.606	0.508	0.580[b]	11
6	512	0.4	0.3	0.4	0.2	0.325[b]	29	0.358	0.543	0.488	0.495	0.471[b]	17

[a]Coefficient of variation (standard deviation X 100/mean).
[b]Significantly different from control (P = 0.05).

TABLE 3. ANALYSIS OF VARIANCE OF SURVIVAL DATA IN TABLE 2.

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	1.575	0.315	13.3[a]	2.77
Within	18	0.426	0.024		
Total	23	2.001			

[a]Significant at P = 0.01.

TABLE 4. RESULTS FROM ANALYSIS OF VARIANCE OF DRY WEIGHT DATA IN TABLE 2.

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	0.155	0.031	9.31[a]	2.77
Within	18	0.060	0.003		
Total	23	0.215			

[a]Significant at P = 0.05

TABLE 5. PRECISION OF THE FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[a]

Test	NOEC (ug/L)	LOEC (ug/L)	Chronic Value (ug/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181
6	256	512	362

[a]For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

SUBJECT: RECOMMENDED DEFINITIVE TEST CONDITIONS FOR FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS

The following draft document was provided by William Peltier, Region IV, U.S. Environmental Protection Agency, in March 1988. The test conditions listed were put into effect in April 1988.

SUBJECT: **RECOMMENDED DEFINITIVE TEST CONDITIONS FOR FATHEAD MINNOW (*PIMEPHALES PROMELAS*) LARVAL SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS**

TABLE 1. SUMMARY OF RECOMMENDED DEFINITIVE TEST CONDITIONS FOR FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS.

1.	Test type:	Static renewal
2.	Temperature (°C): Mean Daily Average	25 ± 1°C
3.	Light quality:	Ambient laboratory illumination
4.	Light intensity:	10-20 uE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod:	16 h light, 8 h dark
6.	Test chamber size:	500 mL containers
7.	Test solution volume:	250 mL/replicate
8.	Renewal of test concentrations:	Daily
9.	Age of test organisms:	1-5 days (48-h old range in age)
10.	Larvae/test chamber and control:	10 larvae/chamber; minimum of 30 larvae/test concentration
11.	Replicate chambers/ concentration:	4 recommended (minimum of 3 acceptable)
12.	Feeding regime:	Feed 0.1 mL 24 h old or less brine shrimp nauplii in a concentrated suspension twice daily. Sufficient nauplii are to be added as to provide an excess for the larvae
13.	Cleaning:	Siphon daily, immediately before test solution renewal
14.	Aeration:	None, unless DO concentration falls below 40% saturation. Rate should be less than 100 bubbles/min.
15.	Dilution water:	Milli Q or equivalent water used to make standard moderately hard water or diluted mineral water (i.e. 9 parts Milli Q and 1 part Perrier)
16.	Effluent concentrations:	Minimum of 5 concentrations and a control
17.	Dilution factor:	0.3
18.	Test duration:	7 days
19.	Effects measured:	Survival and growth (increase in weight)
20.	Test acceptability:	80% or greater survival in the control concentration
21.	Sampling requirement:	Minium of three 24-hr composite samples.1/ Holding times for samples not to exceed 72 h.
22.	Minimum sample volume required daily:	2 L

1/ For a screening test a single 24 h composite sample may be used throughout the test.

SUBJECT: *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

The following method is from a U.S. Environmental Protection Agency document: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, edited by W. B. Horning and C. I. Weber, 1985, pp. 58-75.

SECTION 13

TEST METHOD[1,2]

CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST
METHOD 1002.0

1. SCOPE AND APPLICATION

1.1 This method measures the chronic toxicity of whole effluents and receiving water to the cladoceran, Ceriodaphnia dubia, during a seven-day, static renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 Ceriodaphnia are exposed in a static renewal system for seven days to different concentrations of effluent, or to receiving water. Test results are based on survival and reproduction. If the test is conducted as described, the control organisms should produce three broods of young during the seven-day period.

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

[1]The format used for this method was taken from Kopp, 1983.
[2]This method was adapted from Norberg and Mount, 1984.

4.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4.4 The amount and type of natural food in the effluent or dilution water may confound test results.

4.5 Food added during the test may sequester metals and other toxic substances and confound test results.

5. SAFETY

5.1 See Section 3, Health and Safety.

6. APPARATUS AND EQUIPMENT

6.1 Laboratory *Ceriodaphnia* culture unit -- See culturing methods below. To test effluent or receiving water toxicity, sufficient numbers of newborn (neonate) organisms must be available.

6.2 Samplers -- Automatic sampler, preferrably with sample cooling capability, capable of collecting a 24-h composite sample of 1 L.

6.3 Sample containers -- for sample shipment and storage (See Section 8, Effluent and Receiving Water Sampling and Sample Handling).

6.4 Environmental chamber, incubator, or equivalent facility with temperature control (25$\pm$ 1°C).

6.5 Water purification system -- Millipore Super-Q or equivalent.

6.6 Balance -- Analytical, capable of accurately weighing 0.0001 g.

6.7 Reference weights, Class S -- for checking performance of balance.

6.8 Racks for test vessels -- Racks approximately 8 cm x 40 cm, drilled to hold 10 test vessels each.

6.9 Dissecting microscope -- for examining organisms in the test chambers.

6.10 Light box -- for illuminating organisms during examination.

6.11 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for culture work and preparation of test solutions.

6.12 Volumetric pipets-- Class A, 1-100 mL.

6.13 Serological pipets-- 1-10 mL, graduated.

6.14 Pipet bulbs and fillers -- Propipet[R], or equivalent.

6.15 Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, 2-mm ID -- for transferring organisms.

6.16 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

6.17 Glass or electronic thermometers -- for measuring water temperatures.

6.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.19 National Bureau of Standards Certified thermometer -- see EPA Method 170.1, USEPA 1979b.

6.20 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.21 Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section 5, Facilities and Equipment.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as activated-carbon-filtered distilled or deionized water which does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.5 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling. Dilution water that contains undesirable organisms, that may attack the test organisms should be filtered through a fine mesh net (30-um or smaller openings).

7.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7.6 Laboratory quality assurance samples and standards for the above methods.

7.7 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

7.8 Reference toxicant solutions (see Section 4, Quality Assurance).

7.9 Test Vessels -- 30-mL borosilicate glass beakers or disposable plastic salad dressing cups (manufactured by Anchor-Hocking Plastic Division, and supplied by Plastics Inc., 224 Ryan Avenue, St. Paul, Minnesota, 55164) are recommended because they will fit in the viewing field of most stereoscopic microscopes. Rinse thoroughly in distilled water and then in dilution water before use. A volume of 15 mL of test solution is adequate for the organisms, and will provide a depth in which it is possible to count the animals with a minimum of re-focusing. Ten test vessels are used for each effluent dilution and for the control.

7.10 Test Organisms -- Neonate Ceriodaphnia dubia 2- to 24-h old and released during the same 4-h period. See information on culturing methods below.

7.10.1 The test organism (species being used) cultures should be started at least two weeks before the brood animals are needed, to provide an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

7.10.2 Ceriodaphnia may be shipped or otherwise transported in polyethylene bottles. Several hundred animals will live as long as one week in a 1-L bottle filled three-fourths full with culture medium containing the trout chow diet (Paragraph 7.10.6.2.4 below). Animals received from an outside source should be transferred to new culture media gradually, over a period of 1-2 days, to avoid mass mortality.

7.10.3 It is best to start the culture from one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by preparing slide mounts, regardless of the number of animals used to start the culture. The following procedure is recommended for making slide mounts of Ceriodaphnia (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip.)
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended

mounting medium is CMCP-9/9AF Medium[1], prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fucnsin may be used.

5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1978, and Berner, 1985).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

7.10.4 One-litre glass beakers are recommended for use as culture vessels. Use of aquaria (40- to 80-L, or 10- to 20-gal) and other types of culture vessels may also be convenient. Maintain cultures in several (four or more) separate vessels to provide back-up cultures in case one is lost due to accident or other problems, such as low DO concentrations or lack of food. Fill the 1-L culture vessels with 900 mL of medium.

7.10.5 A new culture is started each week, and the oldest culture is discarded. Using this schedule, 1-L cultures will provide 500 to 1000 neonate Ceriodaphnia per week for use in the tests.

7.10.6 Feeding

7.10.6.1 Feeding the proper amount of the right food is extremely important in Ceriodaphnia culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may clog the animal's filtering apparatus or greatly decrease the DO concentration, and lead to the death of the animals. The suspension of trout chow, yeast, and CEROPHYL^R described below will provide adequate nutrition if fed daily at the rate of 3 mL/L of medium.

7.10.6.2 The combined food is prepared from three ingredients as follows:

7.10.6.2.1 Digested trout chow:

1. Add 5.0 g of No. 1 trout chow, U.S. Fish and Wildlife Service Specification Diet SD9-30, to 1 L of distilled or deionized water. This trout chow may be obtained from Ziegler Bros., Inc., P. O. Box 95, Gardners, PA, 17324. Mix well in a blender and aerate continuously (digest) for one week at ambient laboratory temperature prior to use.

[1]CMCP-9 and 9AF are available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA, 18976. (215-343-6484).

2. At the end of digestion period, place the mixture in a refrigerator and allow to settle overnight. Decant 300 mL of the supernatant and combine with equal volumes of supernatant from CEROPHYL^R and yeast preparations (below). Discard the remainder.

7.10.6.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S^R or ST. REGIS^R, to 1 L of distilled water.
2. Stir with a magnetic stirrer until well dispersed or use a blender at low speed for 5 min.
3. Place in a refrigerator overnite, mix well, and combine 300 mL with equal volumes of supernatant from the trout chow (above) and CEROPHYL^R preparations (below). Discard the remainder.

7.10.6.2.3 CEROPHYL^R (Powdered, Dried, Cereal Leaves)[1]:

1. Place 5.0 g of CEROPHYL^R powder in a blender.
2. Add 1 L of distilled water.
3. Mix at high speed for 5 min.
4. Place in a refrigerator overnite to settle, decant 300 mL of the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard the remainder.

7.10.6.2.4 Combined trout chow-yeast-CEROPHYL^R food:

1. Mix equal (300 mL) volumes of the three foods.
2. Place aliquots of the final mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Fresh or thawed food is stored in the refrigerator between feedings, and is used for a maximum of one week.

7.10.6.2.5 The quality of each batch of food prepared with a new supply of components should be determined by using the food in a 7-day reproduction test with control water (use culture medium, see 7.10.7, below).

7.10.7 Synthetic, moderately hard water (hardness of 80 to 100 mg/L as $CaCO_3$) is recommended as a standard culture medium (see Table 1, Section 7, Dilution Water). Other culture water, such as well water, pond water, or dechlorinated tap water, also may be satisfactory.

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[1] Available from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178. (800-325-3010).

7.10.8 Ceriodaphnia should be cultured at a temperature of 25 ± 2°C No water temperature control equipment is needed if the ambient laboratory temperature remains in the recommended range, and if there are no frequent, rapid, large, temperature excursions in the culture room.

7.10.9 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

7.10.10 Ceriodaphnia cannot survive DO concentrations below 5 mg/L for extended periods. However, aeration is generally not needed unless the cultures are overfed. DO should be measured when the cultures are first started and weekly thereafter. Aerate if the DO concentration drops below 5 mg/L.

7.10.11 Suspend a clear glass or plastic panel over the cultures to exclude dust and dirt. There should be space for circulation of air over the vessels to provide oxygen for the cultures.

7.10.12 Ceriodaphnia are eaten by many species of copepods and shrimp, and cultures must be maintained free of preditors. Natural waters used as culture media should be filtered through a plankton net with 30 um mesh openings.

7.10.13 Ceriodaphnia have been reported to be very sensitive to sudden pH and temperature changes, and care should be taken to limit rapid changes in pH to less than 0.5 units and rapid changes in water temperature to less than 5°C.

7.10.14 The test organisms should be handled carefully and as little as possible so that they are not unnecessarily stressed. They should be transferred from cultures to test vessels, and between test vessels, with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

7.10.15 Culture Maintenance

7.10.15.1 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction in the toxicity tests. Stock cultures which are stressed because they are not adequately fed may produce large number of males and ephippial females. Also, brood females and their offspring may produce few young when used in the test.

1. If food is frozen, remove a bottle of food from the freezer 1 h before feeding time, and allow to thaw.
2. Shake thoroughly.
3. Feed daily at the rate of 4 mL/L of medium.
4. Return unused food mixture to the refrigerator. Do not re-freeze. Discard unused portion after one week.

7.10.15.2 Careful culture maintenance is essential. The population should not be allowed to exceed 1000 animals/L of medium. If necessary, thin the cultures every four or five days after changing the medium to prevent crashes and discourage gametogenesis.

7.10.15.3 The culture medium in each culture vessel should be replaced with fresh medium weekly as follows:

1. Pour about one half (450 mL of the 900 mL) of the contents of a culture vessel into a shallow vessel. A large finger bowl works well.
2. Discard the remainder of the medium and animals unless needed for a test or to start a new culture.
3. Clean the culture vessel by brushing the sides and bottom, or wiping with a clean sponge or paper towel, and rinsing with distilled or deionized water. Each month, the culture vessels should be washed as described in Section 5, (Facilities and Equipment), and air dried.
4. Place about 100 mL of fresh medium in the clean culture vessel.
5. Remove about 100 Ceriodaphnia from the holding vessel (finger bowl) with a pipet, plastic tubing, or by dipping with a small beaker, and transfer them to the fresh medium, along with a small amount of the old medium to provide seed bacteria for the new culture.
6. Carefully add sufficient additional fresh medium to fill the culture vessel.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURE

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, is approximately 1 L. Prepare enough test solution (approximately 600 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

11.2 OBTAINING NEONATES FOR THE TEST

11.2.1 This test method requires neonates 2- to 24-h old (all within 4 h of the same age) to begin the test. To obtain a sufficient number of young which are all less than 4-h old, brood animals containing eggs are placed singly in 30-mL beakers containing 15 mL of media (using the same source of dilution water that will be used in the test), four or five days prior to the initiation of the test. One brood animal is needed for each test vessel that will be used in the test. For example, if five concentrations and a control will be used in a test to begin on a Friday, 60 brood animals are placed in individual beakers on Monday and fed daily, as in the test (see Paragraph 11.6). The brood stock are transferred to fresh media daily, and the young are discarded with the old media. Four hours before the test is to begin, the young are removed from the brood beakers and discarded. In this way, all the young in the brood beakers when the test is ready to start will be less than 4-h old, and are used in the test. Some workers prefer to begin removing and saving young from the brood chambers at 2- or 4-h intervals beginning 8 h before the test is scheduled to start. This makes it possible to use older, but similar aged, young for beginning the test, thus improving chances of good survival of test animals.

11.3 START OF THE TEST

11.3.1 The test should begin as soon as possible, preferably within 24 h of sample collection. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to 25 ± 1°C and maintained at that temperature until portions are added to the dilution water.

11.3.2 Begin the test by randomly placing one neonate in each test beaker. Because of their small size and difficulty in handling, the test chambers are usually placed in racks, 10 to a rack. The position of the test chambers is randomized in the racks at the beginning of the test. On following days, the positions of the racks are randomized.

11.4 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.4.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 uE/m^2/s, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. It is critical that the test water temperature be maintained at 25 ± 1°C to obtain three broods in seven days.

11.5 DISSOLVED OXYGEN

11.5.1 Low DO concentrations may be important when running effluent toxicity tests. However, aeration is not practical for the Ceriodaphnia test. If the DO in the effluent and/or dilution water is low, aerate before preparing the test solutions.

11.6 FEEDING

11.6.1 During the test, the Ceriodaphnia are fed the same diet as used for the cultures. The organisms in the test vessels are fed digested trout chow-yeast-CEROPHYL^R diet daily at a rate of 0.1 mL food suspension/15 mL of test solution.

11.7 TEST SOLUTION RENEWAL

11.7.1 Using a glass or polyethylene dropper, or pipet, transfer each test organism daily to a new test vessel containing 15 mL of freshly-prepared test solution and 0.1 mL of the food suspension. The animals should be released under the surface of the water so that air is not trapped under the carapace.

11.8 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO is measured in each test solution at the beginning of each 24-h exposure period, and at the end of the exposure period in one test vessel at each test concentrations and in the control, after the adult

has been removed and the young have been counted. (Acid must not be added to count the young in that chamber.)

11.8.1.2 Temperature, pH, and conductivity are measured at the beginning of each 24-h exposure period at all test concentrations and in the control.

11.8.1.3 Alkalinity and hardness are measured at the beginning of each 24-h exposure period in 100% effluent and in the control.

11.9 OBSERVATIONS DURING THE TEST

11.9.1 Three broods are usually obtained in the controls in a seven-day test conducted at 25°C. The first brood of two to five young is usually released on the third day of the test, soon after the adults are transferred to fresh test solutions. Successive broods are released every 36 to 48 h thereafter, and may contain 10 to 15 young.

11.9.2 Each day, at the time the organisms are to be transferred to fresh test solutions, determine adult survival and count and record the number of young. First remove the adult to the new test solution. Count any dead young, and then add two drops of 1N hydrochloric acid to the vessel (except the vessel used for DO measurements). The living young die quickly and settle to the bottom of the test vessel where they may be counted with a minimum of effort and error. The young are discarded after counting.

11.9.3 The young are best counted with the aid of a stereomicroscope. The organisms are more easily seen if viewed against a black background. If counts are made without the aid of a stereomicroscope, place the test vessels on a black strip of tape on a light box.

11.9.4 In the absence of toxic substances, young production may exceed 30 per adult. If toxic substances are present, young may develop in the brood pouch of the adults, but may not be released during the exposure period.

11.10 TERMINATION OF THE TEST

11.10.1 Because of the rapid rate of development of Ceriodaphnia, the test must be terminated and all observations completed within ± 2 h of exactly seven days after the initiation of the test. The seven-day test period was selected because each control animal will normally produce three broods, totaling 10 to 30, at a test temperature of 25°C. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods.

11.11 SUMMARY OF TEST CONDITONS

11.11.1 A summary of test conditions is listed in Table 1.

12. CALCULATIONS

12.1 DATA PREPARATION

12.1.1 The number of young produced per adult female is determined by taking the total number of young produced until either the time of death of the adult or the end of the experiment, whichever comes first. An animal that dies before producing young would be included in the analysis, with zero entered as the number of young produced. The subsequent calculation of the mean number of young produced per adult for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction.

12.1.2 Tabulate and summarize the data. A sample set of test data are listed in Table 2. Using these data, the total number of young produced per adult during the test is shown in Table 3.

12.2. DATA ANALYSIS

12.2.1 Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) and Dunnett's Procedure (Dunnett, 1955) or Steel's Many-One Rank Test (Steel, 1959; Miller, 1981) are used to analyze the data. The first step is to test for a significant difference in the survival of the original test organisms in the various effluent concentrations and control, using Fisher's Exact Test. Dunnett's Procedure is then used to analyze the reproduction data from only those effluent concentrations where mortality was not significantly different from the controls.

12.2.2 For the data in Table 3, reference to the tabulated values in Appendix E show that the survival in 25% effluent is significantly different from the control survival. Therefore, the 25% effluent concentration will not be included in the analysis of the reproduction data.

12.2.3 Since it is border-line as to whether the remaining data meet the assumptions for normality, both Dunnett's Procedure and Steel's Many-One Rank Test can be used to analyze the reproduction data.

12.2.4 The safe concentration derived from the test is reported as the NOEC.

12.2.5 Dunnett's Procedure

12.2.5.1 Dunnett's procedure includes an analysis of variance (ANOVA) followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means. An example of a Dunnett's calculation is included in the Appendix. The computer program listed in the Appendix generates output which includes an ANOVA table, a statement about each treatment mean that can be used to identify the NOEC, and the minimum difference between treatment and control means that can be detected as statistically significant.

12.2.5.2 The analysis is carried out using the total number of live young produced by each adult female during the the test (Table 3).

12.2.5.3 The output from the analysis of variance is shown in Table 4, and indicates that the effects of the various treatments on reproduction were significant at P = 0.05.

12.2.5.4 The computer output for the comparison of the control mean with the treatment means is shown below:

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2 (1% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3 (3% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4 (6% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5 (12% EFFLUENT) AND CONTROL.

12.2.5.5 The NOEC and LOEC, as determined from the analysis, were 12% and 25% effluent, respectively. Note that the Dunnett program considers the control as Concentration 1. Thus, Concentration 5 in the output from Dunnett's test is effluent Concentration 4 (12% effluent) in the test.

12.2.5.6 For this set of data, the minimum difference that can be detected as statistically significant is 6.28 young per adult. This represents a 34.5% reduction in the response from the control.

12.2.6 Steel's Many-One Rank Test

12.2.6.1 Steel's Many-One Rank Test compares several treatments with a control by analyzing the ranks of the data, thereby eliminating the necessity that the data meet the normality assumptions. The calculations for Steel's Many-One Rank Test of the data in Table 3 are detailed in the Appendix. The NOEC determined from Steel's Test was 12%, which agrees with the NOEC from Dunnett's Procedure above.

12.2.7 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

Log_{10} NOEC = Log_{10} 12 = 1.0792
Log_{10} LOEC = Log_{10} 25 = 1.3979
ChV = Antilog (1.0792 + 1.3979)/2 = Antilog 1.2386
ChV = 17.3% effluent

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Information on the single laboratory precision of the Ceriodaphnia reproduction test based on the NOEC and LOEC values from nine tests with the reference toxicant NaPCP is provided in Table 5.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 1. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST

1. Test type:	Static renewal
2. Temperature (^{o}C):	25 $\pm$ 1°C
3. Light quality:	Ambient laboratory light
4. Light intensity:	10-20 uE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test vessel size:	30 mL
7. Test solution volume:	15 mL
8. Renewal of test concentrations:	Daily
9. Age of test organisms:	Less than 24 h; and all released within a 4-h period
10. Number of test organisms per chamber:	1
11. Number of replicate chambers per treatment:	10
12. Feeding regime:	Feed 0.1 mL food suspension/15 mL, daily
13. Aeration:	None
14. Dilution water:	Moderately hard standard water, receiving water, other surface water, or ground water with hardness similar to receiving water
15. Dilution factor:	Approximately 0.3 or 0.5
16. Test duration:	7 days
17. Effects measured:	Survival and reproduction

SUBJECT: *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

TABLE 2. DATA FROM CERIODAPHNIA EFFLUENT TOXICITY TEST

Effl Conc	Day No.	Replicate A	B	C	D	E	F	G	H	I	J	Total Live Young	No. Live Adults	Most Young By Any One Adult
Cont	3	0	0	0	0	0	x	0	0	0	0	0	9	0
	4	2	2	4	0	6	-	2	6	1	4	27	9	6
	5	9	2	9	0	9	-	0	6	2	9	46	9	9
	6	5	6	9	6	0	-	3	5	11	10	55	9	11
	7	6	8	5	10	1	-	3	8	10	3	54	9	10
		22	18	27	16	16	0	8	25	24	26	182		
1.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	2	4	1	0	4	2	2	3	2	2	22	10	4
	5	9	5	2	0	8	13	2	8	2	2	51	10	13
	6	6	3	6	2	8	8	10	0	6	6	55	10	10
	7	5	3	12	4	3	8	9	0	13	12	69	10	13
		22	15	21	6	23	31	23	11	23	22	197		
3.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	2	1	2	4	4	8	2	0	4	3	30	10	8
	5	3	4	2	9	11	6	2	4	8	6	55	10	11
	6	10	8	6	9	0	3	6	10	8	3	63	10	10
	7	10	12	10	2	0	6	11	8	0	6	65	10	12
		25	25	20	24	15	23	21	22	20	18	213		
6.0%	3	0	0	0	0	0	0	0	0	0	3	3	10	3
	4	2	4	2	2	0	1	2	0	4	2	19	10	4
	5	2	8	0	2	4	2	2	4	6	8	38	10	8
	6	9	2	2	3	10	6	8	6	2	4	52	10	10
	7	10	2	3	6	12	13	9	11	9	4	79	10	13
		23	16	7	13	26	22	21	21	21	21	191		
12.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	0	4	2	2	2	0	1	2	1	4	18	10	4
	5	1	8	0	2	3	3	0	2	3	0	22	10	8
	6	8	4	8	3	10	10	5	10	6	7	71	10	10
	7	11	5	7	6	10	12	10	11	8	7	87	10	12
		20	21	17	13	25	25	16	25	18	18	198		
25.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	x	0	0	0	x	x	0	x	0	0	0	6	0
	5	-	x	1[x]	x	-	-	x	-	x	x	1	1	1
	6	-	-	-	-	-	-	-	-	-	-	0	0	0
	7	-	-	-	-	-	-	-	-	-	-	0	0	0
		0	0	1	0	0	0	0	0	0	0	1		

x = Dead adult, no young produced before death.
1[x] = Dead adult; one young produced before death.

Note: Days 1 and 2 are not included because young were not produced until the third day. Adult mortality was not recorded for days 1 and 2.

TABLE 3. NUMBER OF YOUNG PRODUCED PER CERIODAPHNIA

Replicate (Organism)	Control	Effluent Concentration (%) 1	3	6	12	25
A	22	22	25	23	20	0
B	18	15	25	16	21	0
C	27	21	20	7	17	1
D	16	6	24	13	13	0
E	16	23	15	26	25	0
F	0	31	23	22	25	0
G	8	23	21	21	16	0
H	25	11	22	21	25	0
I	24	23	20	21	18	0
J	26	22	18	21	18	0

TABLE 4. ANALYSIS OF VARIANCE OF CERIODAPHNIA REPRODUCTION DATA FROM CONTROL AND FIRST FOUR EFFLUENT CONCENTRATIONS (1-12%)

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	4	51.480	12.870	0.35	2.58
Within	45	1664.300	36.984		
TOTAL	49	1715.780			

TABLE 5. PRECISION OF THE CERIODAPHNIA REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[a]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[b]	0.25	0.50	0.35
2[c]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[d]	0.30	0.60	0.42
5	0.30	0.60	0.42
6	0.30	0.60	0.42
7	0.30	0.60	0.42
8	0.30	0.60	0.42
9	0.30	0.60	0.42

[a]For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

[b]Concentrations used in Test 1 were; 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

[c]Concentrations used in Tests 2 and 3 were, 0.007, 0.022, 0.067, 0.20, 0.60 mg NaPCP/L.

[d]Concentrations used in Tests 4 through 9 were, 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

SUBJECT: **RECOMMENDED DEFINITIVE TEST CONDITIONS FOR *CERIODAPHNIA* SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS**

The following draft document was provided by William Peltier, Region IV, U.S. Environmental Protection Agency, in March 1988. The test conditions listed were put into effect in April 1988.

SUBJECT: **RECOMMENDED DEFINITIVE TEST CONDITIONS FOR *CERIODAPHNIA* SURVIVAL AND GROWTH TEST USED FOR NPDES PERMITS**

TABLE 1. SUMMARY OF RECOMMENDED DEFINITIVE TEST CONDITIONS FOR CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST USED FOR NPDES PERMITS.

1.	Test type:	Static renewal
2.	Temperature (°C): Mean Daily Average	25 ± 1°C
3.	Light quality:	Ambient laboratory light
4.	Light intensity:	10-20 uE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5.	Photoperiod:	16 h light, 8 h dark
6.	Test chamber size:	30 mL
7.	Test solution volume:	15 mL
8.	Renewal of test concentrations:	Daily
9.	Age of test organisms:	Less than 24h, all released within a 6-h period
10.	Number of test organisms per chamber:	1
11.	Number of replicate chambers per treatment:	10
12.	Feeding regime:	Feed 0.1 mL each of YCT and Selenastrum capricornutum suspension/15 mL daily. Alga suspension of 31-34 x 10^6 cells/mL
13.	Aeration:	None
14.	Dilution water:	Milli Q or equivalent water used to make standard moderately hard water or diluted mineral water (i.e. 9 parts Milli Q and 1 part Perrier)
15.	Effluent concentrations:	Minimum of 5 concentrations and a control
16.	Dilution factor:	0.3
17.	Test duration:	7 days or until 60% of control females have had their 3rd brood
18.	Effects measured:	Survival and reproduction
19.	Test acceptability:	80% or greater survival and an average of 15 young/female in the control concentration
20.	Sampling requirement:	Minimum of three 24 h composite samples. 1/ Holding times for samples not to exceed 72 h
21.	Minimum sample volume required daily:	500 mL

1/ For a screening test a single 24 h composite sample maybe used throughout the test.

SUBJECT: **INDEX OF KEY WORDS**

Brine shrimp, SOP-5, 6, 9

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    culture, SOP-1, 3, 4, 10, 15; REF-2, 3, 9, 10
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