SECTION 13

TEST METHOD

DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST METHOD 1002.0

13.1 SCOPE AND APPLICATION

13.1.1 This method measures the chronic toxicity of effluents and receiving water to the daphnid, *Ceriodaphnia dubia*, using less than 24 h old neonates during a three-brood (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.

13.1.2 Daily observations on mortality make it possible to also calculate acute toxicity for desired exposure periods (i.e., 24-h, 48-h, and 96-h LC50s).

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

13.1.4 Brief excursions in 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 degradable or highly volatile toxicants in the source may not be detected in the test.

13.1.5 This test method is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

13.2 SUMMARY OF METHOD

13.2.1 *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent, or to receiving water, until 60% or more of surviving control females have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the surviving control organisms should produce 15 or more young in three broods. If these criteria are not met at the end of 8 days, the test must be repeated.

13.3 INTERFERENCES

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

13.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

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

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

13.3.5 Food added during the test may sequester metals and other toxic substances and confound test results. Daily renewal of solutions, however, will reduce the probability of reduction of toxicity caused by feeding.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the initial pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

13.3.6.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 13.3.6.1.1).

13.3.6.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the CO_2-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992);

or by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio or the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, CO_2 is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.4 SAFETY

13.4.1 See Section 3, Health and Safety.

13.5 APPARATUS AND EQUIPMENT

13.5.1 *Ceriodaphnia* and algal culture units -- See *Ceriodaphnia* and algal culturing methods below and algal culturing methods in Section 14 and USEPA, 2002a.

13.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, capable of collecting a 24-h composite sample of 5 L or more.

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

13.5.4 Environmental chambers, incubators, or equivalent facilities with temperature control (25 ± 1 °C).

13.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

13.5.6 Balance -- analytical, capable of accurately weighing 0.00001 g.

13.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the material to be weighed.

13.5.8 Test chambers -- 10 test chambers are required for each concentration and control. Test chambers such as 30-mL borosilicate glass beakers or disposable polystyrene cups are recommended because they will fit in the viewing field of most stereoscopic microscopes. The glass beakers and plastic cups are rinsed thoroughly with dilution water before use. To avoid potential contamination from the air and excessive evaporation of the test solutions during the test, the test vessels should be covered with safety glass plates or sheet plastic (6 mm thick).

13.5.9 Mechanical shaker or magnetic stir plates -- for algal cultures.

13.5.10 Light meter -- with a range of 0-200 $\mu E/m^2/s$ (0-1000 ft-c).

13.5.11 Fluorometer (optional) -- equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

13.5.12 UV-VIS spectrophotometer (optional) -- capable of accommodating 1-5 cm cuvettes.

13.5.13 Cuvettes for spectrophotometer -- 1-5 cm light path.

13.5.14 Electronic particle counter (optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

13.5.15 Microscope with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope) -- for determining sex and verifying identification.

13.5.16 Dissecting microscope, stereoscopic, with zoom objective, magnification to 50X -- for examining and counting the neonates in the test vessels.

13.5.17 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

13.5.18 Centrifuge (optional) -- plankton, or with swing-out buckets having a capacity of 15-100 mL.

13.5.19 Centrifuge tubes -- 15-100 mL, screw-cap.

13.5.20 Filtering apparatus -- for membrane and/or glass fiber filters.

13.5.21 Racks (boards) -- to hold test chambers. It is convenient to use a piece of styrofoam insulation board, 50 cm x 30 cm x 2.5 cm (20 in x 12 in x 1 in), drilled to hold 60 test chambers, in six rows of 10 (see Figure 1).

13.5.22 Light box -- for illuminating organisms during examination.

13.5.23 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.

13.5.24 Pipettors, adjustable volume repeating dispensers -- for feeding. Pipettors such as the Gilson REPETMAN®, Eppendorf, Oxford, or equivalent, provide a rapid and accurate means of dispensing small volumes (0.1 mL) of food to large numbers of test chambers.

13.5.25 Volumetric pipets -- class A, 1-100 mL.

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

13.5.27 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

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

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

13.5.30 Thermometer, glass or electronic, laboratory grade, -- for measuring water temperatures.

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

13.5.32 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

13.5.33 Meters, DO, pH, and specific conductivity -- for routine physical and chemical measurements.

13.6 REAGENTS AND CONSUMABLE MATERIALS

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

13.6.2 Data sheets (one set per test) -- for recording the data.

13.6.3 Vials, marked -- for preserving specimens for verification (optional).

13.6.4 Tape, colored -- for labeling test vessels.

13.6.5 Markers, waterproof -- for marking containers.

13.6.6 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA, 1979b.

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

13.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA, 1979b.

13.6.9 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

13.6.10 Laboratory quality control samples and standards -- for calibration of the above methods.

13.6.11 Reference toxicant solutions -- see Section 4, Quality Assurance.

13.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

13.6.13 Effluent, surface water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.6.14 Trout chow, yeast, and CEROPHYL® food (or substitute food) -- for feeding the cultures and test organisms.

13.6.14.1 Digested trout chow, or substitute flake food (TETRAMIN®, BIORIL®, or equivalent), is prepared as follows:

1. Preparation of trout chow or substitute flake food requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife Service specifications.
2. Add 5.0 g of trout chow pellets or substitute flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.
3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

13.6.14.2 Yeast is prepared as follows:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® Yeast, Lake State Kosher Certified Yeast, or equivalent, to 1 L of MILLI-Q® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

13.6.14.3 CEROPHYLL® is prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves, CEROPHYLL®, or equivalent are available from commercial sources. Dried, powdered, alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet shops.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

13.6.14.4 Combined yeast-cerophyl-trout chow (YCT) is mixed as follows:

1. Thoroughly mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks. Do not store frozen over three months.
4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7-1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

13.6.15 Algal food -- for feeding the cultures and test organisms.

13.6.15.1 Algal Culture Medium is prepared as follows:

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L, mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.
3. Immediately filter the medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 mL MILLI-Q® WATER	
1. MACRONUTRIENTS			
A.	$MgCl_2 \cdot 6H_2O$	6.08	g
	$CaCl_2 \cdot 2H_2O$	2.20	g
	$NaNO_3$	12.75	g
B.	$MgSO_4 \cdot 7H_2O$	7.35	g
C.	K_2HPO_4	0.522	g
D.	$NaHCO_3$	7.50	g
2. MICRONUTRIENTS			
	H_3BO_3	92.8	mg
	$MnCl_2 \cdot 4H_2O$	208.0	mg
	$ZnCl_2$	1.64	mg[1]
	$FeCl_3 \cdot 6H_2O$	79.9	mg
	$CoCl_2 \cdot 6H_2O$	0.714	mg[2]
	$Na_2MoO_4 \cdot 2H_2O$	3.63	mg[3]
	$CuCl_2 \cdot 2H_2O$	0.006	mg[4]
	$Na_2EDTA \cdot 2H_2O$	150.0	mg
	Na_2SeO_4	1.196	mg[5]

[1] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[2] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[3] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.

[4] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.

[5] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (mg/L)	ELEMENT	CONCENTRATION (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µg/L)	ELEMENT	CONCENTRATION (µg/L)
H_3BO_3	185.0	B	32.5
$MnCl_2 \cdot 4H_2O$	416.0	Mn	115.0
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.0	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.0	--	----
Na_2SeO_4	2.39	Se	0.91

13.6.15.2 Algal Cultures

13.6.15.2.1 See Section 6, Test Organisms, for information on sources of "starter" cultures of *Selenastrum capricornutum, S. minutum*, and *Chlamydomonas reinhardti.*

13.6.15.2.2 Two types of algal cultures are maintained: "stock" cultures, and "food" cultures.

13.6.15.2.2.1 Establishing and Maintaining Stock Cultures of Algae:

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia dubia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia dubia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained at 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms (see Section 6, Test Organisms) every four to six months.

13.6.15.2.2.2 Establishing and Maintaining "Food" Cultures of Algae:

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia dubia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$ or 400 ft-c).
3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

13.6.15.2.3 Preparing Algal Concentrate for Use as *Ceriodaphnia dubia* Food:

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer (see Section 14, Green Alga, *Selenastrum capricornutum* Growth Test), and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia dubia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

13.6.15.3 Food Quality

13.6.15.3.1 USEPA recommends Fleishmann's® yeast, Cerophyll®, trout chow, and *Selenastrum capricornutum* as the preferred *Ceriodaphnia dubia* food combination. This recommendation is based on extensive data developed by many laboratories which indicated high *Ceriodaphnia dubia* survival and reproduction in culturing and testing. The use of substitute food(s) is acceptable only after side-by-side tests are conducted to determine that the quality of the substitute food(s) is equal to the USEPA recommended food combination based on survival and reproduction of *Ceriodaphnia dubia*.

13.6.15.3.2 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, algae, and/or any substitute food(s) should be determined in side-by-side comparisons of *Ceriodaphnia dubia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

13.6.16 TEST ORGANISMS, DAPHNIDS, *CERIODAPHNIA DUBIA*

13.6.16.1 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

13.6.16.2 Neonates used for toxicity tests must be obtained from individually cultured organisms. Mass cultures may be maintained, however, to serve as a reserve source of organisms for use in initiating individual cultures and in case of loss of individual cultures.

13.6.16.3 Starter animals may be obtained from commercial sources and may be shipped in polyethylene bottles. Approximately 40 animals and 3 mL of food are placed in a l-L bottle filled full with culture water for shipment. 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.

13.6.16.4 It is best to start the cultures with one animal, which is sacrificed after producing young, mounted on a microscope slide, and retained as a permanent 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 dubia* (modified from 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-9/10 Medium, prepared by mixing two parts of CMCP-9 with one part of CMCP-10 stained with enough acid fuchsin dye to color the mixture a light pink. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.
5. Using forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a 12 mm round 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 varnish around the edges of the coverslip.
9. Identify to species (see Pennak, 1978; Pennak, 1989; and Berner, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

13.6.16.5 Mass Culture

13.6.16.5.1 Mass cultures are used only as a "backup" reservoir of organisms.

13.6.16.5.2 One-liter or 2-L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

13.6.16.5.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal with new culture medium at least twice a week for two weeks. At each renewal, the adult survival is recorded, and the offspring and the old medium are discarded. After two weeks, the adults are also discarded, and the culture is re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia dubia* each week.

13.6.16.6 Individual Culture

13.6.16.6.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

13.6.16.6.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests (see Figure 1).

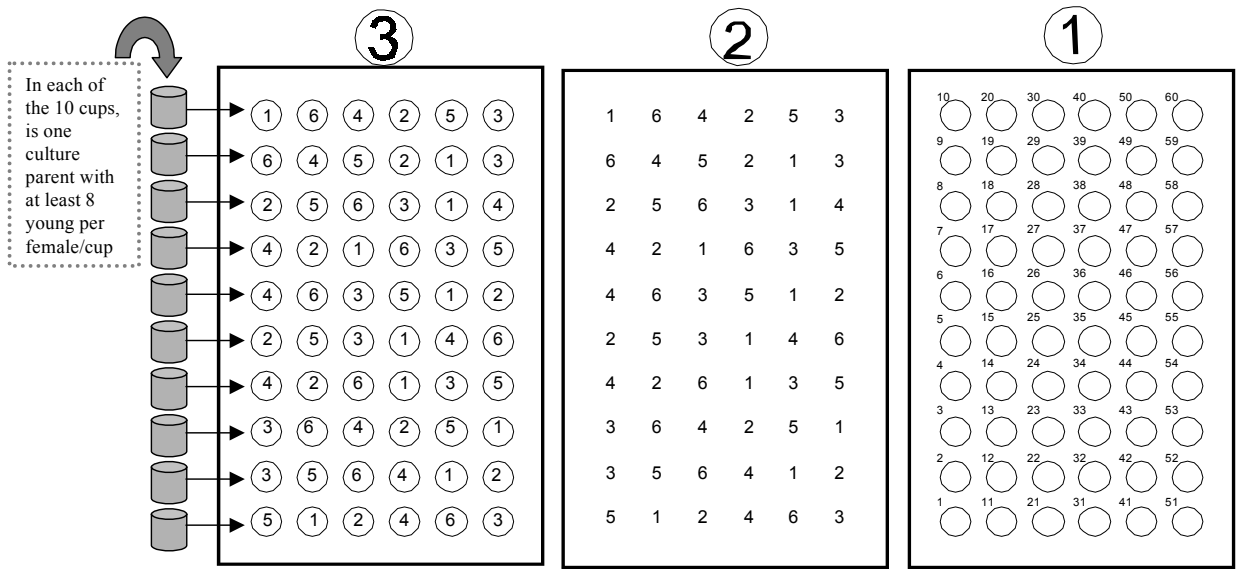

Figure 1. Examples of a test board and randomizing template: 1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets, 2) cardboard randomizing template prepared by randomly drawing numbers (1-6) for each position in a row across the board, and 3) test board (1) placed on top of the randomizing template (2) for the purpose of assigning the position of test treatments (1-6) within each block (row on the test board). Following placement of test chambers, test organisms are allocated using blocking by known parentage. Test organisms from a single brood cup are distributed to each treatment within a given block (row on the test board).

13.6.16.6.3 Organisms are fed daily (see Subsection 13.6.16.9) and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

13.6.16.6.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

13.6.16.6.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

13.6.16.6.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults, or less than an average of 20 young per female would indicate problems, such as poor quality of culture media or food. Cultures that do not meet these criteria should not be used as a source of test organisms.

13.6.16.7 Culture Medium

13.6.16.7.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

13.6.16.8 Culture Conditions

13.6.16.8.1 The daphnid, *Ceriodaphnia dubia,* should be cultured at a temperature of 25 ± 1°C.

13.6.16.8.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A photoperiod of 16-h of light and 8-h of darkness is recommended. Light intensity should be 10-20 $\mu E/m^2/s$ or 50 to 100 ft-c.

13.6.16.8.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

13.6.16.8.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred 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.

13.6.16.9 Food and Feeding

13.6.16.9.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia dubia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT), along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

13.6.16.9.2 Other algal species (such as *S. minutum* or *Chlamydomonas reinhardti*), other substitute food combinations (such as Flake Fish Food), or different feeding rates may be acceptable as long as performance criteria are met and side-by-side comparison tests confirm acceptable quality (see Subsection 13.6.15.3).

13.6.16.9.3 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low

numbers of young, large numbers of males, and/or ephippial females. Also, their offspring may produce few young when used in toxicity tests.

13.6.16.9.4 Feed as follows:

1. If YCT is frozen, remove a bottle of food from the freezer 1h before feeding time, and allow to thaw.
2. YCT food mixture and algal concentrates should both be thoroughly mixed by shaking before dispensing.
3. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
4. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after two weeks.

13.6.16.10 It is recommended that chronic toxicity tests be performed monthly with a reference toxicant. Daphnid, *Ceriodaphnia dubia,* neonates less than 24 h old, and all within 8 h of the same age are used to monitor the chronic toxicity of the reference toxicant to the *Ceriodaphnia dubia* produced by the culture unit (see Section 4, Quality Assurance).

13.6.16.11 Record Keeping

13.6.16.11.1 Records, kept in a bound notebook, include (1) source of organisms used to start the cultures, (2) type of food and feeding times, (3) dates culture were thinned and restarted, (4) rate of reproduction in individual cultures, (5) daily observations of the condition and behavior of the organisms in the cultures, and (6) dates and results of reference toxicant tests performed (see Section 4, Quality Assurance).

13.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

13.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

13.8 CALIBRATION AND STANDARDIZATION

13.8.1 See Section 4, Quality Assurance.

13.9 QUALITY CONTROL

13.9.1 See Section 4, Quality Assurance.

13.10 TEST PROCEDURES

13.10.1 TEST SOLUTIONS

13.10.1.1 Receiving Waters

13.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. For a test consisting of single receiving water and control, approximately 600 mL of sample would be required for each test, assuming 10 replicates of 15 mL, and sufficient additional sample for chemical analysis.

13.10.1.2 Effluents

13.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5, and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

13.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

13.10.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, with a dilution series of 0.5, is approximately 1 L/day. 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 under a stereomicroscope with a minimum of re-focusing. Ten test chambers are used for each effluent dilution and for the control. Sufficient test solution (approximately 550 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses at the high, medium, and low test concentrations.

13.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

13.10.1.2.5 Just prior to test initiation (approximately one h) the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature and maintained at that temperature during the preparation of the test solutions.

13.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO concentration below 4.0 mg/L, all the solutions and the control must be gently aerated.

13.10.1.3 Dilution Water

13.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

13.10.2 START OF THE TEST

13.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have ten replicates.

13.10.2.2 The test chambers must be randomly assigned to a board using a template (Figure 1) or by using random numbers (see Appendix A). Randomizing the position of test chambers as described in Figure 1 (or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4). A number of different templates should be prepared, and the template used for each test should be identified on the data sheet. The same template must not be used for every test.

13.10.2.3 Neonates less than 24 h old, and all within 8 h of the same age, are required to begin the test. The neonates must be obtained from individual cultures using brood boards, as described above in Subsection 13.6.16.6,

Individual Culture (also see Section 6, Test Organisms). Neonates must be taken only from adults in individual cultures that have eight or more young in their third or subsequent broods. These adults can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 mL YCT and 0.1 mL algal concentrate/15 mL of media). Record the age range of test organisms, source, and feeding of neonates on test data sheets.

13.10.2.4 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber in the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". The technique used to achieve blocking by known parentage should be recorded in the test data report. One effective technique is to block randomize the test board as described in Figure 1 and transfer one neonate from the first brood cup to each of the six test chambers in the first row on the test board. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 60 test chambers contains one neonate. The set of six test chambers (one for each test treatment) containing organisms derived from a single female parent is referred to as a block. When using the technique described in Figure 1, each row of the test board will represent a block.

13.10.2.4.1 The brood cups and test chambers may be placed on a light table to facilitate counting the neonates. However, care must be taken to avoid temperature increase due to heat from the light table.

13.10.2.4.2 Following the allocation of test organisms to the test board, additional neonates might remain in the ten brood cups that were selected for test setup. These additional neonates may be discarded, used as future culture organisms if needed, or used to start additional tests (provided that at least 6 neonates remain and these neonates continue to meet test organism age requirements).

13.10.2.5 Blocking by known parentage allows the performance of each test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. Also, by knowing the parentage of each test organism, blocks consisting largely of males can be omitted from all test treatments at the end of the test (see Subsection 13.13.1.4), decreasing variability among replicates.

13.10.3 LIGHT, PHOTOPERIOD, AND TEMPERATURE

13.10.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 $\mu E/m^2/s$, or 50 to 100 ft-c, with a photoperiod of 16 h of light and 8 h of darkness.

13.10.3.2 It is critical that the test water temperature be maintained at 25 ± 1°C to obtain three broods in seven days.

13.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.4.1 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 be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the test solutions on subsequent days. The DO concentration should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). Aeration is generally not practical during the daphnid, *Ceriodaphnia dubia*, test. If the DO in the effluent and/or dilution water is low, aerate gently before preparing the test solutions. 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, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the organisms.

13.10.5 FEEDING

13.10.5.1 The organisms are fed when the test is initiated, and daily thereafter. Food is added to the fresh medium immediately before or immediately after the adults are transferred. Each feeding consists of 0.1 mL YCT and 0.1 mL *Selenastrum capricornutum* concentrate/15 mL test solution (0.1 mL of algal concentrate containing 3.0-3.5 X 10^7 cells/mL will provide 2-2.3 X 10^5 cells/mL in the test chamber).

13.10.5.2 The YCT and algal suspension can be added accurately to the test chambers by using automatic pipettors, such as Gilson, Eppendorf, Oxford, or equivalent.

13.10.6 OBSERVATIONS DURING THE TEST

13.10.6.1 Routine Chemical and Physical Determinations

13.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentration and in the control.

13.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in sufficient number of test vessels at least at the end of the test to determine the temperature variation in the environmental chamber.

13.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

13.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

13.10.6.1.5 Record the data on data sheet (Figure 2).

13.10.6.2 Routine Biological Observations

13.10.6.2.1 Three or four broods are usually obtained in the controls in a 7-day test conducted at 25 ± 1°C. A brood is a group of offspring released from the female over a short period of time when the carapace is discarded during molting. In the controls, the first brood of two-to-five young is usually released on the third or fourth day of the test. Successive broods are released every 30 to 36 h thereafter. The second and third broods usually consist of eight to 20 young each. The total number of young produced by a healthy control organism in three broods often exceeds 30 per female. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.6.2.2 The release of a brood may be inadvertently interrupted during the daily transfer of organisms to fresh test solutions, resulting in a split in the brood count between two successive days. For example, four neonates of a brood of five might be released on Day 3, just prior to test solution renewal, and the fifth released just after renewal, and counted on Day 4. Partial broods, released over a two-day period, should be counted as one brood.

13.10.6.2.3 Each day, the live adults are transferred to fresh test solutions, and the numbers of live young are recorded (see data form, Figure 3). The young can be counted with the aid of a stereomicroscope with substage lighting. Place the test chambers on a light box over a strip of black tape to aid in counting the neonates. The young are discarded after counting.

13.10.6.2.4 Some of the effects caused by toxic substances include, (1) a reduction in the number of young produced, (2) young may develop in the brood pouch of the adults, but may not be released during the exposure

period, and (3) partially or fully developed young may be released, but are all dead at the end of the 24-h period. Such effects should be noted on the data sheets (Figure 3).

13.10.6.2.5 Protect the daphnids, *Ceriodaphnia dubia*, from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and transfer of females carefully. Make sure the females remain immersed during the performance of these operations.

13.10.7 DAILY PREPARATION OF TEST CHAMBERS

13.10.7.1 The test is started (Day 0) with new disposable polystyrene cups or precleaned 30-mL borosilicate glass beakers that are labeled and color-coded with tape. Each following day, a new set of plastic cups or precleaned glass beakers is prepared, labeled, and color-coded with tape similar to the original set. New solutions are placed in the new set of test chambers, and the test organisms are transferred from the original test chambers to the new ones with corresponding labels and color-codes. Each day, previously used glass beakers are recleaned (see Section 5, Facilities, Equipment, and Supplies) for the following day, and previously used plastic cups are discarded.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations.

Discharger:_____ Test Dates:_____

Location:_____ Analyst:_____

Control:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Conc:		Day 1	2	3	4	5	6	7	Remarks
Temp.									
D.O.	Initial								
	Final								
pH	Initial								
	Final								
Alkalinity									
Hardness									
Conductivity									
Chlorine									

Figure 2. Data form for the daphnid, *Ceriodaphnia dubia,* survival and reproduction test. Routine chemical and physical determinations (CONTINUED).

13.10.8 TEST SOLUTION RENEWAL

13.10.8.1 Freshly prepared solutions are used to renew the test daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. No more than 36 h should elapse between collection of the sample and the first use in the test. Maintain the samples in the refrigerator at 0-6°C until used.

13.10.8.2 New test solutions are prepared daily, and the test organisms are transferred to the freshly prepared solutions using a small-bore (2 mm) glass or polyethylene dropper or pipet. The animals are released under the surface of the water so that air is not trapped under the carapace. Organisms that are dropped or injured are discarded.

13.10.9 TERMINATION OF THE TEST

13.10.9.1 Tests should be terminated when 60% or more of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. Because of the rapid rate of development of *Ceriodaphnia dubia*, at test termination all observations on organism survival and numbers of offspring should be completed within two hours. 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. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test.

13.10.9.2 Count the young, conduct required chemical measurements, and complete the data sheets (Figure 3).

13.10.9.3 Any animal not producing young should be examined to determine if it is a male (Berner, 1986). In most cases, the animal will need to be placed on a microscope slide before examining (see Subsection 13.6.16.4).

13.10.9.3.1 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of the *Ceriodaphnia dubia* Survival and Reproduction Test, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress (Pennak, 1989), culture conditions should be examined whenever males are identified in a test.

13.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

13.11.1 A summary of test conditions and test acceptability criteria is presented in Table 3.

13.12 ACCEPTABILITY OF TEST RESULTS

13.12.1 For the test results to be acceptable, at least 80% of all control organisms must survive, and 60% of surviving control females must produce at least three broods, with an average of 15 or more young per surviving female.

Discharger:_____ Analyst:_____
Location: _____ Test Start-Date/Time: _____
Date Sample Collected: _____ Test Start-Date/time: _____

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data.

Discharger:_____ Analyst:_____
Location: _____ Test Start-Date/Time: _____
Date Sample Collected: _____ Test Start-Date/time: _____

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

	Replicate										Number of Young	Number of Adults	Young per Adult
	1	2	3	4	5	6	7	8	9	10			
Conc.	Day												
	1												
	2												
	3												
	4												
	5												
	6												
	7												
	Total												

Figure 3. Data form for the daphnid, *Ceriodaphnia dubia*, survival and reproduction test. Daily summary of data (CONTINUED).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0)[1]

1.	Test type:	Static renewal (required)
2.	Temperature (°C):	25 ± 1°C (recommended) Test temperatures should not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
3.	Light quality:	Ambient laboratory illumination (recommended)
4.	Light intensity:	10-20 µE/m^2/s, or 50-100 ft-c (ambient laboratory levels) (recommended)
5.	Photoperiod:	16 h light, 8 h dark (recommended)
6.	Test chamber size:	30 mL (recommended minimum)
7.	Test solution volume:	15 mL (recommended minimum)
8.	Renewal of test solutions:	Daily (required)
9.	Age of test organisms:	Less than 24 h; and all released within a 8-h period (required)
10.	No. neonates per test chamber:	1 Assigned using blocking by known parentage (Subsection 13.10.2.4) (required)
11.	No. replicate test chambers per concentration:	10 (required minimum)
12.	No. neonates per test concentration:	10 (required minimum)
13.	Feeding regime:	Feed 0.1 mL each of YCT and algal suspension per test chamber daily (recommended)
14.	Cleaning:	Use freshly cleaned glass beakers or new plastic cups daily (recommended)
15.	Aeration:	None (recommended)
16.	Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water) (available options)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1002.0) (CONTINUED)

17.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving Water: 100% receiving water (or minimum of 5) and a control (recommended)
18.	Dilution factor:	Effluents: ≥ 0.5 (recommended) Receiving Waters: None or ≥ 0.5 (recommended)
19.	Test duration:	Until 60% or more of surviving control females have three broods (maximum test duration 8 days) (required)
20.	Endpoints:	Survival and reproduction (required)
21.	Test acceptability criteria:	80% or greater survival of all control organisms and an average of 15 or more young per surviving female in the control solutions. 60% of surviving control females must produce three broods (required)
22.	Sampling requirements:	For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
23.	Sample volume required:	1 L/day (recommended)

13.13 **DATA ANALYSIS**

13.13.1 GENERAL

13.13.1.1 Tabulate and summarize the data. A sample set of survival and reproduction data is listed in Table 4.

TABLE 4. SUMMARY OF SURVIVAL AND REPRODUCTION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, EXPOSED TO AN EFFLUENT FOR SEVEN DAYS

Effluent Concentration (%)	No. of Young per Adult Replicate										No. Live Adults
	1	2	3	4	5	6	7	8	9	10	
Control	27	30	29	31	16	15	18	17	14	27	10
1.56	32	35	32	26	18	29	27	16	35	13	10
3.12	39	30	33	33	36	33	33	27	38	44	10
6.25	27	34	36	34	31	27	33	31	33	31	10
12.5	10	13	7	7	7	10	10	16	12	2	10
25.0	0	0	0	0	0	0	0	0	0	0	2

13.13.1.2 The endpoints of toxicity tests using the daphnid, *Ceriodaphnia dubia*, are based on the adverse effects on survival and reproduction. The LC50, the IC25, the IC50 and the EC50 are calculated using point estimation techniques, and LOEC and NOEC values for survival and reproduction are obtained using a hypothesis test approach such as Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962), Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of the LC50, IC25, IC50 and EC50. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for reproduction, but included in the estimation of the LC50, IC25, IC50, and EC50. See the Appendices for examples of the manual computations, program listings, and examples of data input and program output.

13.13.1.3 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 Appendix B. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

13.13.1.4 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproduction endpoint (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of the survival endpoint (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of survival (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). Note that the exclusion of males from the analysis of reproduction may create unequal sample sizes among the concentrations, influencing the statistical methods chosen for analysis of reproduction (Figure 6). Determinations regarding test acceptability criteria for survival and reproduction (Subsection 13.12) must be made prior to exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the

control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample.

13.13.2 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL DATA

13.13.2.1 Formal statistical analysis of the survival data is outlined on the flowchart in Figure 4. The response used in the analysis is the number of animals surviving at each test concentration. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC50, LC50, IC25, or IC50 endpoints. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the LC, EC, and IC endpoints.

13.13.2.2 Fisher's Exact Test is used to determine the NOEC and LOEC endpoints. It provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli (binomial) population. Additional information on Fisher's Exact Test is provided in Appendix G.

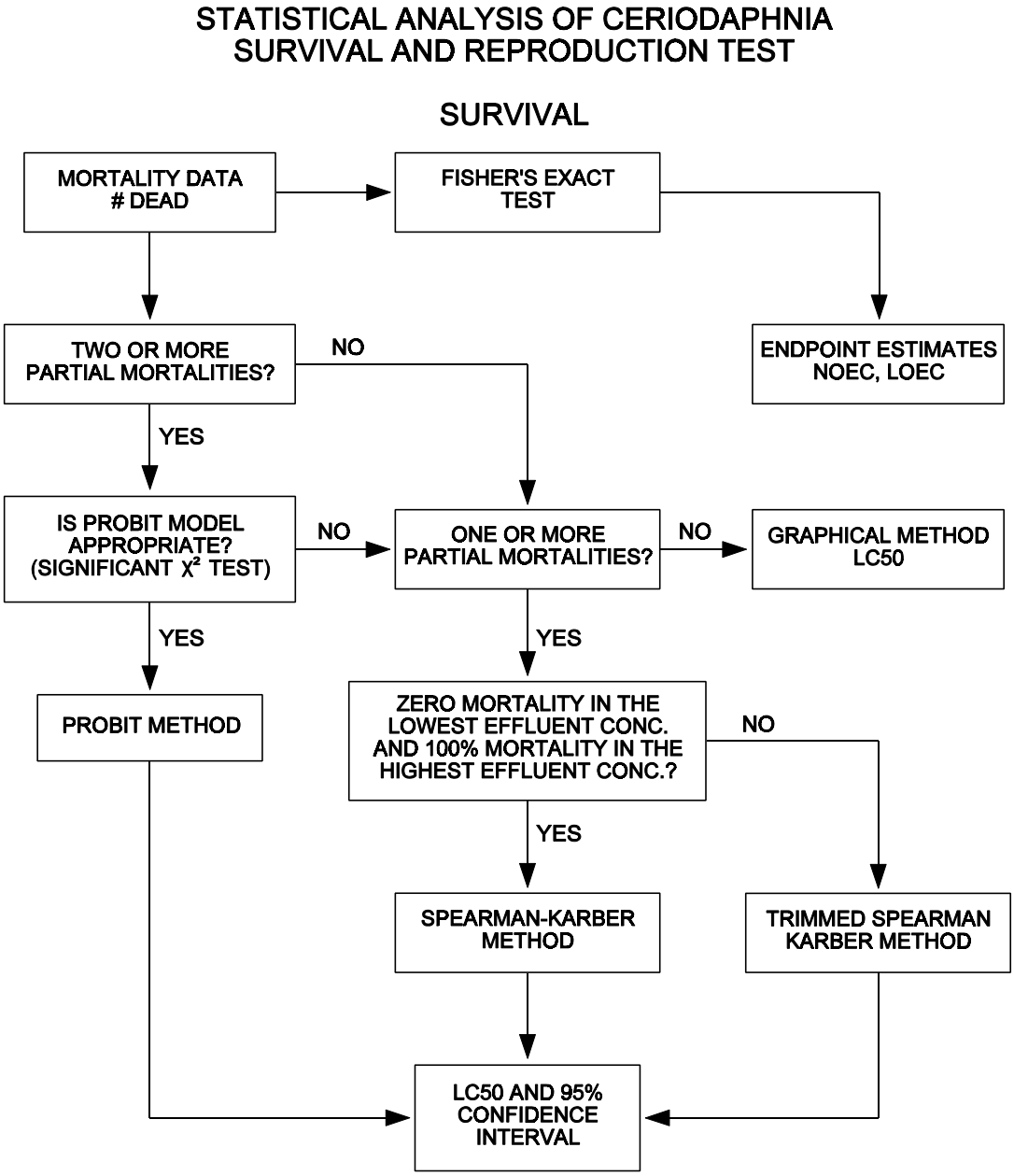

Figure 4. Flowchart for statistical analysis of the daphnid, *Ceriodaphnia dubia*, survival data.

13.13.2.3 Probit Analysis (Finney, 1971; Appendix I) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total number dead at a given concentration is the response.

13.13.2.4 Example of Analysis of Survival Data

13.13.2.4.1 The data in Table 4 will be used to illustrate the analysis of survival data from the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test. As can be seen from the data in Table 4, there were no deaths in the 1.56%, 3.12%, 6.25%, and 12.5% concentrations. These concentrations are obviously not different from the control in terms of survival. This leaves only the 25% effluent concentration to be tested statistically for a difference in survival from the control.

13.13.2.5 Fisher's Exact Test

13.13.2.5.1 The basis for Fisher's Exact Test is a 2x2 contingency table. From the 2x2 table prepared by comparing the control and the effluent concentration, determine statistical significance by looking up a value in the table provided in Appendix G (Table G.5). However, to use this table the contingency table must be arranged in the format illustrated in Table 5.

TABLE 5. FORMAT OF THE 2x2 CONTINGENCY TABLE

	Number of		Number of
	Successes	Failures	Observations
Condition 1	a	A - a	A
Condition 2	b	B - b	B
Total	a + b	[(A+B) - a - b]	A + B

13.13.2.5.2 Arrange the table so that the total number of observations for row one is greater than or equal to the total for row two ($A \geq B$). Categorize a success such that the proportion of successes for row one is greater than or equal to the proportion of successes for row two ($a/A \geq b/B$). For these data, a success may be 'alive' or 'dead' whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is included in Appendix G, Table G.5. Enter Table G.5 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05 in Table G.5, then the survival proportion for the effluent concentration is significantly different from that of the control. A dash or absence of entry in Table G.5 indicates that no contingency table in that class is significant.

13.13.2.5.3 To compare the control and the effluent concentration of 25%, the appropriate contingency table for the test is given in Table 6.

13.13.2.5.4 Since $10/10 \geq 3/10$, the category 'alive' is regarded as a success. For A = 10, B = 10 and, a = 10, under the column headed 0.05, the value from Table G.5 is b = 6. Since the value of b (b = 3) from the contingency table (Table 6), is less than the value of b (b = 6) from Table G.5 in Appendix G, the test concludes that the proportion

surviving in the 25% effluent concentration is significantly different from the control. Thus the NOEC for survival is 12.5% and the LOEC is 25%.

TABLE 6. 2x2 CONTIGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		
	Alive	Dead	Number of Observations
Condition 1	10	0	10
Condition 2	3	7	10
Total	13	7	20

13.13.2.6 Calculation of the LC50

13.13.2.6.1 The data used for the Trimmed Spearman-Karber Method are summarized in Table 7. To perform the Trimmed Spearman-Karber Method, run the USEPA Trimmed Spearman-Karber Program. An example of the program input and output is supplied in Appendix J.

TABLE 7. DATA FOR TRIMMED SPEARMAN-KARBER ANALYSIS

	Effluent Concentration (%)					
	Control	1.56	3.12	6.25	12.5	25.0
Number Dead	0	0	0	0	0	8
Number Exposed	10	10	10	10	10	10

13.13.2.6.2 For this example, with only one partial mortality, Trimmed Spearman-Karber analysis appears appropriate for this data.

13.13.2.6.3 Figure 5 shows the output for the Trimmed Spearman-Karber Analysis of the data in Table 7 using the USEPA Program.

13.13.3 EXAMPLE OF ANALYSIS OF THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

13.13.3.1 Formal statistical analysis of the reproduction data is outlined on the flowchart in Figure 6. The response used in the statistical analysis is the number of young produced per adult female, which 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. In this three-brood test, offspring from fourth or higher broods should not be counted and should not be included in the total number of neonates produced during the test. An animal that dies before

producing young, if it has not been identified as a male, would be included in the analysis with zero entered as the number of young produced. The subsequent calculation of the mean number of live young produced per adult female for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction. An IC estimate can be calculated for the reproduction data using a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain an NOEC for reproduction. Concentrations above the NOEC for survival are excluded from the hypothesis test for reproduction effects.

13.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, normality and homogeneity of variance, are formally tested using the Shapiro Wilk's Test for normality, and Bartlett's Test for homogeneity of variance. If either of these tests fails, a nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

13.13.3.3 Additionally, if unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

13.13.3.4 The data, mean, and variance of the observations at each concentration including the control are listed in Table 8. A plot of the number of young per adult female for each concentration is provided in Figure 7. Since there is significant mortality in the 25% effluent concentration, its effect on reproduction is not considered.

TABLE 8. THE DAPHNID, *CERIODAPHNIA DUBIA,* REPRODUCTION DATA

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	27	32	39	27	10
2	30	35	30	34	13
3	29	32	33	36	7
4	31	26	33	34	7
5	16	18	36	31	7
6	15	29	33	27	10
7	18	27	33	33	10
8	17	16	27	31	16
9	14	35	38	33	12
10	27	13	44	31	2
Mean $\overline{Y}_I$	22.4	26.3	34.6	31.7	9.4
S_i^2	48.0	64.0	23.4	8.7	15.1
i	1	2	3	4	5

TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

DATE: 1 TEST NUMBER: 2 DURATION: 7 Days
TOXICANT: effluent
SPECIES: *Ceriodaphnia dubia*

RAW DATA:

Concentration (%)	Number Exposed	Mortalities
.00	10	0
1.25	10	0
3.12	10	0
6.25	10	0
12.5	10	0
25.0	10	8

SPEARMAN-KARBER TRIM: 20.00 %

SPEARMAN-KARBER ESTIMATES: LC50: 19.28
95% CONFIDENCE LIMITS
ARE NOT RELIABLE.

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

Figure 5. Output for USEPA Trimmed Spearman-Karber program.

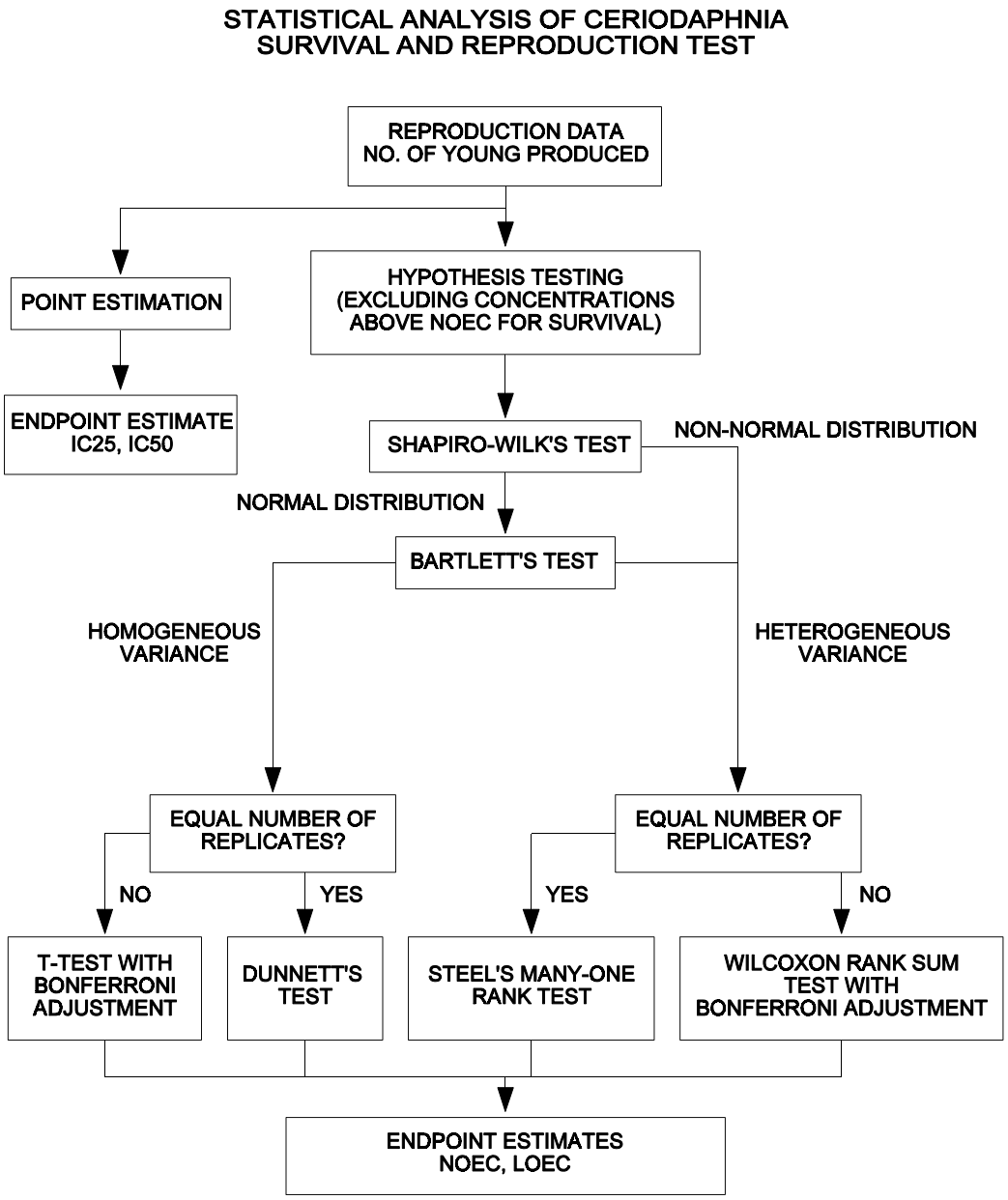

Figure 6. Flowchart for the statistical analysis of the daphnid, *Ceriodaphnia dubia*, reproduction data.

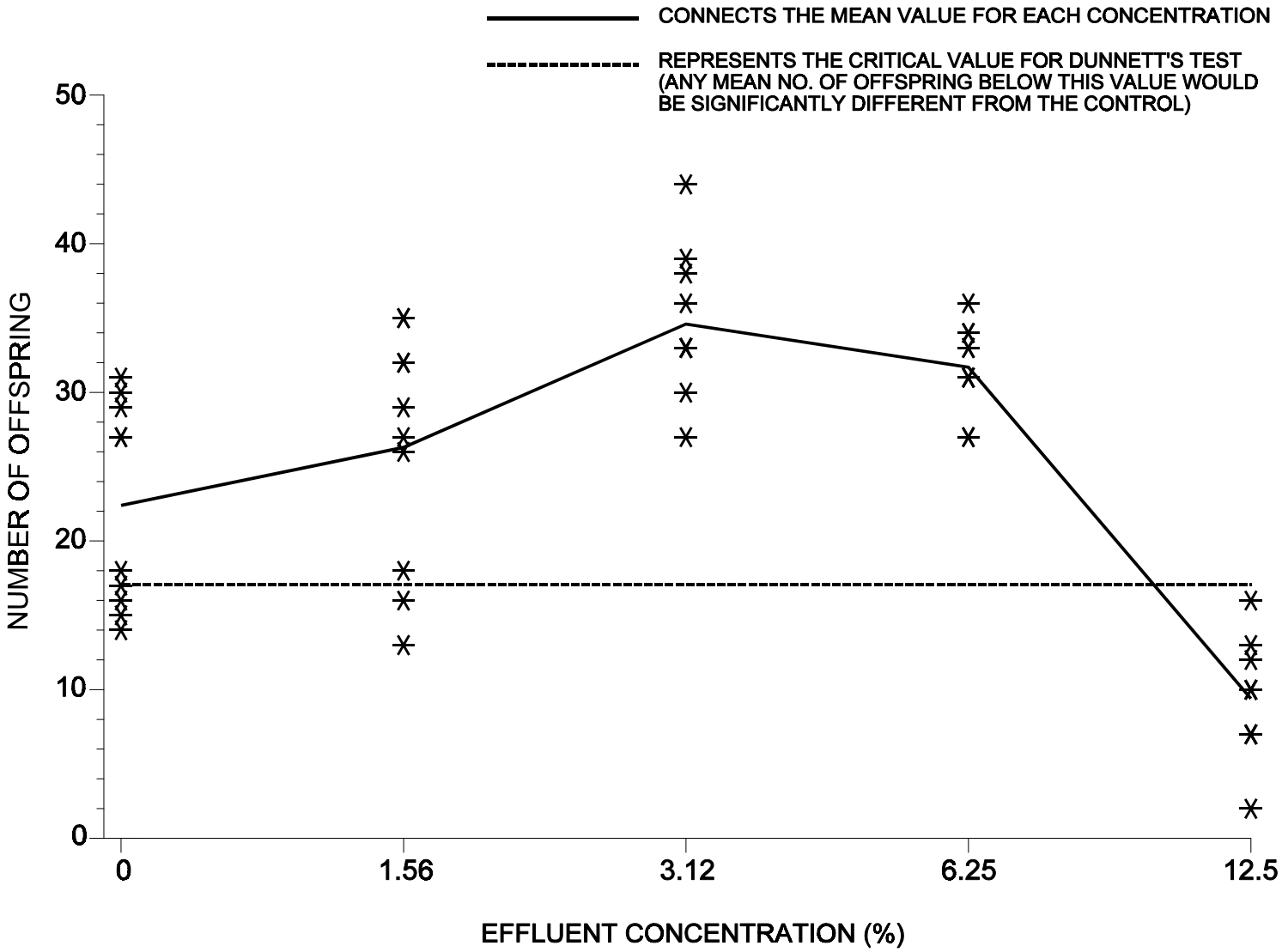

Figure 7. Plot of number of young per adult female from a daphnid, *Ceriodaphnia dubia*, survival and reproduction test.

13.13.3.5 Test for Normality

13.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 9.

TABLE 9. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)			
Replicate	Control	1.56	3.12	6.25	12.5
1	4.6	5.7	4.4	-4.7	0.6
2	7.6	8.7	-4.6	2.3	3.6
3	6.6	5.7	-1.6	4.3	-2.4
4	8.6	-0.3	-1.6	2.3	-2.4
5	-6.4	-8.3	1.4	-0.7	-2.4
6	-7.4	2.7	-1.6	-4.7	0.6
7	-4.4	0.7	-1.6	1.3	0.6
8	-5.4	-10.3	-7.6	-0.7	6.6
9	-8.4	8.7	3.4	1.3	2.6
10	4.6	-13.3	9.4	-0.7	-7.4

13.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the <u>overall</u> mean of the centered observations

n = the total number of centered observations.

For this set of data,

$$n = 50$$

$$\bar{X} = \frac{1}{50}(0.0) = 0.0$$

$$D = 1433.4$$

13.13.3.5.3 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 10.

13.13.3.5.4 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 50, k = 25. The a_i values are listed in Table 11.

13.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 11.

For this set of data:

$$W = \frac{1}{1433.4}(37.3)^2 = 0.97$$

TABLE 10. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-13.3	26	0.6
2	-10.3	27	0.6
3	-8.4	28	0.7
4	-8.3	29	1.3
5	-7.6	30	1.3
6	-7.4	31	1.4
7	-7.4	32	2.3
8	-6.4	33	2.3
9	-5.4	34	2.6
10	-4.7	35	2.7
11	-4.7	36	3.4
12	-4.6	37	3.6
13	-4.4	38	4.3
14	-2.4	39	4.4
15	-2.4	40	4.6
16	-2.4	41	4.6
17	-1.6	42	5.7
18	-1.6	43	5.7
19	-1.6	44	6.6
20	-1.6	45	6.6
21	-0.7	46	7.6
22	-0.7	47	8.6
23	-0.7	48	8.7
24	-0.3	49	8.7
25	0.6	50	9.4

TABLE 11. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3751	22.7	$X^{(50)} - X^{(1)}$
2	0.2574	19.0	$X^{(49)} - X^{(2)}$
3	0.2260	17.1	$X^{(48)} - X^{(3)}$
4	0.2032	16.9	$X^{(47)} - X^{(4)}$
5	0.1847	15.2	$X^{(46)} - X^{(5)}$
6	0.1691	14.0	$X^{(45)} - X^{(6)}$
7	0.1554	14.0	$X^{(44)} - X^{(7)}$
8	0.1430	12.1	$X^{(43)} - X^{(8)}$
9	0.1317	11.1	$X^{(42)} - X^{(9)}$
10	0.1212	9.3	$X^{(41)} - X^{(10)}$
11	0.1113	9.3	$X^{(40)} - X^{(11)}$
12	0.1020	9.0	$X^{(39)} - X^{(12)}$
13	0.0932	8.7	$X^{(38)} - X^{(13)}$
14	0.0846	6.0	$X^{(37)} - X^{(14)}$
15	0.0764	5.8	$X^{(36)} - X^{(15)}$
16	0.0685	5.1	$X^{(35)} - X^{(16)}$
17	0.0608	4.2	$X^{(34)} - X^{(17)}$
18	0.0532	3.9	$X^{(33)} - X^{(18)}$
19	0.0459	3.9	$X^{(32)} - X^{(19)}$
20	0.0386	3.0	$X^{(31)} - X^{(20)}$
21	0.0314	2.0	$X^{(30)} - X^{(21)}$
22	0.0244	2.0	$X^{(29)} - X^{(22)}$
23	0.0174	1.4	$X^{(28)} - X^{(23)}$
24	0.0104	0.9	$X^{(27)} - X^{(24)}$
25	0.0035	0.0	$X^{(26)} - X^{(25)}$

13.13.3.5.6 The decision rule for this test is to compare W with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 50 observations (n) is 0.930. Since W = 0.97 is greater than the critical value, the conclusion of the test is that the data are normally distributed.

13.13.3.6 Test for Homogeneity of Variance

13.13.3.6.1 The test used to examine whether the variation in number of young produced is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ln \overline{S}^2 - \sum_{i=1}^{P} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration and control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{P} V_i S_i^2)}{\sum_{i=1}^{P} V_i}$$

$$C = 1 + (3(p-1))^{-1}[\sum_{i=1}^{P} \frac{1}{V_i} - (\sum_{i=1}^{P} V_i)^{-1}]$$

13.13.3.6.2 For the data in this example (see Table 8), all effluent concentrations including the control have the same number of replicates (n_i = 10 for all i). Thus, V_i = 9 for all i.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(45)\ln(31.8) - 9\sum_{i=1}^{P} \ln(S_i^2)]/1.04$$

$$= [45(3.46) - 9(16.061)]/1.04$$

$$= 11.15/1.04$$

$$= 10.72$$

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = 10.7 is less than the critical value of 13.3, conclude that the variances are not different.

13.13.3.7 Dunnett's Procedure

13.13.3.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 12.

TABLE 12. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	= SSB/(p-1)
Within	N - p	SSW	= SSW/(N-p)
Total	N - 1	SST	

Where: p = number effluent concentrations including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$ Between Sum of Squares

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$ Total Sum of Squares

$$SSW = SST - SSB$$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{P} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the number of young produced by female j in effluent concentration i)

13.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = 10$

N = 50

$T_1 = Y_{11} + Y_{12} + \ldots + Y_{110} = 224$
$T_2 = Y_{21} + Y_{22} + \ldots + Y_{210} = 263$

$T_3 = Y_{31} + Y_{32} + \ldots + Y_{310} = 346$
$T_4 = Y_{41} + Y_{42} + \ldots + Y_{410} = 317$
$T_5 = Y_{51} + Y_{52} + \ldots + Y_{510} = 94$

$G = T_1 + T_2 + T_3 + T_4 + T_5 = 1244$

$$SSB = \sum_{i=1}^{P} \frac{T_i^2}{n_i} - \frac{G^2}{N}$$

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

$$SST = \sum_{i=1}^{P}\sum_{j=1}^{n_i} Y_{ij}^2 - \frac{G^2}{N}$$

$$= 36{,}272 - \frac{(1244)^2}{50} = 5321.28$$

$$SSW = SST - SSB = 5321.28 - 3887.88 = 1433.40$$

S_B^2 = SSB/(p-1) = 3887.88/(5-1) = 971.97

S_W^2 = SSW/(N-p) = 1433.40/(50-5) = 31.85

13.13.3.7.3 Summarize these calculations in an ANOVA table (Table 13).

TABLE 13. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	4	3887.88	971.97
Within	45	1433.40	31.85
Total	49	5321.28	

13.13.3.7.4 To perform the individual comparisons, calculate the t statistic for each concentration and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(\frac{1}{n_1}) + (\frac{1}{n_i})}}$$

Where: $\bar{Y}_i$ = mean number of young produced for effluent concentration i

$\bar{Y}_1$ = mean number of young produced for the control

S_W = square root of within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i.

Since we are looking for a decrease in reproduction from the control, the mean for concentration i is subtracted from the control mean in the t statistic above. However, if we were looking for an increased response over the control, the control mean would be subtracted from the mean at a concentration.

13.13.3.7.5 Table 14 includes the calculated t values for each concentration and control combination. In this example, comparing the 1.56% concentration with the control the calculation is as follows:

$$t_2 = \frac{(22.4 - 26.3)}{[5.64\sqrt{(\frac{1}{10}) + (\frac{1}{10})}]} = -1.55$$

TABLE 14. CALCULATED T VALUES

Effluent Concentration (%)	i	t_i
1.56	2	-1.55
3.12	3	-4.84
6.25	4	-3.69
12.5	5	5.16

13.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean reproduction, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. Since an entry for 45 degrees of freedom for error is not provided in the table, the entry for 40 degrees of freedom for error, an alpha level of 0.05 and four concentrations (excluding the control) will be used, 2.23. The mean reproduction for concentration "i" is considered significantly less than the mean reproduction for the control if t_i is greater than the critical value.

Since t_5 is greater than 2.23, the 12.5% concentration has significantly lower reproduction than the control. Hence the NOEC and the LOEC for reproduction are 6.25% and 12.5%, respectively.

13.13.3.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\, S_w \sqrt{(\frac{1}{n_1}) + (\frac{1}{n})}$$

Where: d = the critical value for the Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

13.13.3.7.8 In this example:

$$MSD = 2.23(5.64)\sqrt{(\frac{1}{10})+(\frac{1}{10})}$$

$$= 2.23\ (5.64)\ (0.447)$$

$$= 5.62$$

13.13.3.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 5.62.

13.13.3.7.10 This represents a 25% decrease in mean reproduction from the control.

13.13.3.8 Calculation of the IC

13.13.3.8.1 The reproduction data in Table 4 are utilized in this example. As can be seen from Figure 8, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

13.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1$= 22.4, and the observed mean for the lowest effluent concentration,$\overline{Y}_2$= 26.3, we see that $\overline{Y}_1$ is less than $\overline{Y}_2$.

13.13.3.8.3 Calculate the smoothed means:

$$M_1 = M_2 = (\overline{Y}_1+\overline{Y}_2)/2 = 24.35$$

13.13.3.8.4 Since $\overline{Y}_3$= 34.6 is larger than M_2, average $\overline{Y}_3$ with the previous concentrations:

$$M_1 = M_2 = M_3 = (M_1 + M_2 + \overline{Y}_3)/3 = 27.7.$$

13.13.3.8.5 Additionally, $\overline{Y}_4$ = 31.7 is larger than M_3, and is pooled with the first three means. Thus:

$$(M_1 + M_2 + M_3 + \overline{Y}_4)/4 = 28.7 = M_1 = M_2 = M_3 = M_4$$

13.13.3.8.6 Since $M_4 > \overline{Y}_5$ = 9.4, set M_5 = 9.4. Likewise, $M_5 > \overline{Y}_6$ = 0, and M_6 becomes 0. Table 15 contains the smoothed means and Figure 8 gives a plot of the smoothed means and the interpolated response curve.

TABLE 15. DAPHNID, *CERIODAPHNIA DUBIA*, REPRODUCTION MEAN RESPONSE AFTER SMOOTHING

Response Effluent Conc. (%)	i	Smoothed Means, Y_i (young/female)	Means, M_i (young/female)
Control	1	22.4	28.75
1.56	2	26.3	28.75
3.12	3	34.6	28.75
6.25	4	31.7	28.75
12.5	5	9.4	9.40
25.0	6	0.0	0.00

13.13.3.8.7 Estimates of the IC25 and IC50 can be calculated using the Linear Interpolation Method. A 25% reduction in reproduction, compared to the controls, would result in a mean reproduction of 21.56 young per adult, where $M_1(1 - p/100) = 28.75(1 - 25/100)$. A 50% reduction in reproduction, compared to the controls, would result in a mean reproduction of 14.38 young per adult, where $M_1(1 - p/100) = 28.75(1 - 50/100)$. Examining the smoothed means and their associated concentrations (Table 15), the two effluent concentrations bracketing 21.56 young per adult are C_4 = 6.25% effluent and C_5 = 12.5% effluent. The two effluent concentrations bracketing a response of 14.38 young per adult are also C_4 = 6.25% and C_5 = 12.5%.

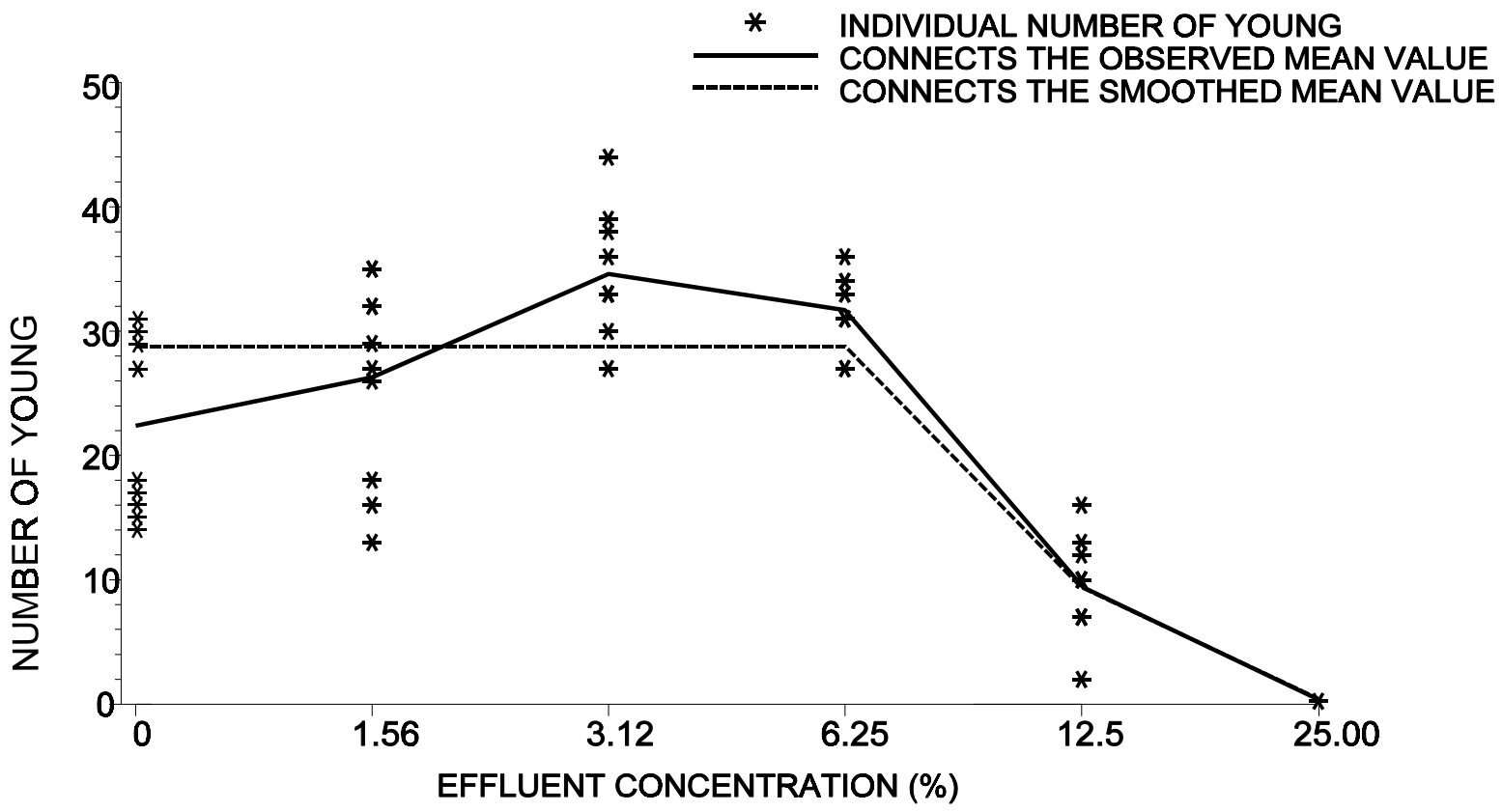

Figure 8. Plot of raw data, observed means, and smoothed means for the daphnid, *Ceriodaphnia dubia*, reproductive data.

13.13.3.8.8 Using equation from Section 4.2 in Appendix M, the estimate of the IC25 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 6.25 + [28.75(1 - \frac{25}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 8.57% effluent

13.13.3.8.9 The estimate of the IC50 is as follows:

$$ICp = C_j + [M_1(1 - \frac{p}{100}) - M_j]\frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 6.25 + [28.75(1 - \frac{50}{100}) - 28.75]\frac{(12.5 - 6.25)}{(9.40 - 28.75)}$$

= 10.89% effluent

13.13.3.8.10 When the ICPIN program was used to analyze this data set for the IC25, requesting 80 resamples, the estimate of the IC25 was 8.5715% effluent. The empirical 95% confidence interval for the true mean was 8.3112% and 9.0418% effluent. The computer output for this data set is provided in Figure 9.

13.13.3.8.11 When the ICPIN program was used to analyze this data set for the IC50, requesting 80 resamples, the estimate of the IC50 was 10.8931% effluent. The empirical 95% confidence interval for the true mean was 10.4373% and 11.6269% effluent. The computer output for this data set is provided in Figure 10.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 8.5715 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.5891 Standard Deviation: 0.1831
Original Confidence Limits: Lower: 8.3112 Upper: 9.0418
Resampling time in Seconds: 2.53 Random Seed: -641671986

Figure 9. Example of ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	1.56	3.12	6.25	12.5	25.0
Response 1	27	32	39	27	10	0
Response 2	30	35	30	34	13	0
Response 3	29	32	33	36	7	0
Response 4	31	26	33	34	7	0
Response 5	16	18	36	31	7	0
Response 6	15	29	33	27	10	0
Response 7	18	27	33	33	10	0
Response 8	17	16	27	31	16	0
Response 9	14	35	38	33	12	0
Response 10	27	13	44	31	2	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date: Example Test Ending Date:
Test Species: Ceriodaphnia dubia
Test Duration: 7-d
DATA FILE: cdmanual.icp
OUTPUT FILE: cdmanual.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Std. Dev.	Pooled Response Means
1	10	0.000	22.400	6.931	28.750
2	10	1.560	26.300	8.001	28.750
3	10	3.120	34.600	4.835	28.750
4	10	6.250	31.700	2.946	28.750
5	10	12.500	9.400	3.893	9.400
6	10	25.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 10.8931 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 10.9316 Standard Deviation: 0.3357
Original Confidence Limits: Lower: 10.4373 Upper: 11.6269
Resampling time in Seconds: 2.58 Random Seed: 172869646

Figure 10. Example of ICPIN program output for the IC50.

13.14 **PRECISION AND ACCURACY**

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Information on the single-laboratory precision of the daphnid, *Ceriodaphnia dubia*, survival and reproduction test is based on the NOEC and LOEC values from nine tests with the reference toxicant sodium pentachlorophenate (NaPCP) is provided in Table 16. The NOECs and LOECs of all tests fell in the same concentration range, indicating maximum possible precision. Table 17 gives precision data for the IC25 and IC50 values for seven tests with the reference toxicant NaPCP. Coefficient of variation was 41% for the IC25 and 28% for the IC50.

13.14.1.1.2 Ten sets of data from six laboratories met the acceptability criteria, and were statistically analyzed using nonparametric procedures to determine NOECs and LOECs.

13.14.1.1.3 EPA evaluated within-laboratory precision of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test using a database of routine reference toxicant test results from 33 laboratories (USEPA, 2000b). The database consisted of 393 reference toxicant tests conducted in 33 laboratories using a variety of reference toxicants including: cadmium, copper, potassium chloride, sodium chloride, and sodium pentachlorophenate. Among the 33 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 reproduction endpoint. In 25% of laboratories, the within-laboratory CV was less than 17%; and in 75% of laboratories, the within-laboratory CV was less than 45%.

TABLE 16: SINGLE LABORATORY PRECISION OF THE DAPHNID, *CERIODAPHNIA DUBIA*, SURVIVAL AND REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[1,2]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[3]	0.25	0.50	0.35
2[4]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[5]	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

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

2 Data from Tests performed by Philip Lewis, Aquatic Biology Branch, EMSL-Cincinnati, OH. Tests were conduted in reconstituted hard water (hardness = 180 mg $CaC0_3$/L; pH - 8.1).

3 Concentrations used in Test 1 were: 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

4 Concentrations used in Tests 2 and 3 were: 0.007, 0.022, 0.067, 0.020, 0.60 mg NaPCP/L.

5 Concentrations used in Tests 4 through 9 were: 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

TABLE 17. THE DAPHNID, *CERIODAPHNIA DUBIA,* SEVEN-DAY SURVIVAL AND REPRODUCTION TEST PRECISION FOR A SINGLE LABORATORY USING NAPCP AS THE REFERENCE TOXICANT (USEPA, 1991a)

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
19	0.30	0.3754	0.4508
46A	0.20	0.0938	0.2608
46B	0.20	0.2213	0.2879
49	0.20	0.2303	0.2912
55	0.20	0.2306	0.3177
56	0.10	0.2241	0.2827
n	7	7	7
Mean	NA	0.2157	0.2953
CV(%)	NA	41.1	27.9

13.14.1.2 Multilaboratory Precision

13.14.1.2.1 A multilaboratory study was performed by the Aquatic Biology Branch, EMSL-Cincinnati in 1985e, involving a total of 11 analysts in 10 different laboratories (Neiheisel et. al., 1988; USEPA, 1988e). Each analyst performed one-to-three seven-day tests using aliquots of a copper-spiked effluent sample, for a total of 25 tests. The tests were performed on the same day in all participating laboratories, using a pre-publication draft of Method 1002.0. The NOECs and LOECs for these tests were within one concentration interval which, with a dilution factor of 0.5, is equivalent to a two-fold range in concentration (Table 18).

13.14.1.2.2 A second multilaboratory study of Method 1002.0 (using the first edition of this manual; USEPA, 1985c), was coordinated by Battelle, Columbus Division, and involved 11 participating laboratories (Table 19) (DeGraeve et al., 1989). All participants used 10% DMW (10% PERRIER® Water) as the culture and dilution water, and used their own formulation of food for culturing and testing the *Ceriodaphnia dubia*. Each laboratory was to conduct at least one test with each of eight blind samples. Each test consisted of 10 replicates of one organism each for five toxicant concentrations and a control. Of the 116 tests planned, 91 were successfully initiated, and 70 (77%) met the survival and reproduction criteria for acceptability of the results (80% survival and nine young per initial female). If the reproduction criteria of 15 young/female, used in this edition of the method, had been applied to the results of the interlaboratory study, 22 additional tests would have been unacceptable. The overall precision (CV) of the test was 27% for the survival data (7-day LC50s) and 37.5% and 39.0% for the reproduction data (IC50s and IC25s, respectively).

13.14.1.2.3 In 2000, EPA conducted an interlaboratory variability study of the daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 3.70%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 20 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 35.0% for IC25 results. Table 21 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant and effluent sample types and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 97.2%, 91.3%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type, three concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 18. INTERLABORATORY PRECISION FOR THE DAPHNID, *CERIODAPHNIA DUBIA,* SURVIVAL AND REPRODUCTION TEST WITH COPPER SPIKED EFFLUENT (USEPA, 1988e)

		Endpoints (% Effluent)			
		Reproduction		Survival	
Analyst	Test	NOEC	LOEC	NOEC	LOEC
3	1	12	25	25	50
4	1	6	12	12	25
4	2	6	12	25	50
5	1	6	12	12	25
5	2	12	25	12	25
6	1	12	25	25	50
6	2	6	12	25	50
10	1	6	12	12	25
10	2	6	12	12	25
11	1	12	25	25	50

TABLE 19. INTERLABORATORY PRECISION DATA FOR THE DAPHNID, *CERIODAPHNIA DUBIA*, SUMMARIZED FOR EIGHT REFERENCE TOXICANTS AND EFFLUENTS (USEPA, 1991a)

Test Material	Mean IC50	CV%	Mean IC25	CV%
Sodium chloride	1.34	29.9	1.00	34.3
Industrial	3.6	83.3	3.2	78.1
Sodium chloride	0.96	57.4	0.09	44.4
Pulp and Paper	60.0	28.3	47.3	27.0
Potassium dichromate	35.8	30.8	23.4	32.7
Pulp and Paper	70.2	7.5	55.7	12.2
Potassium dichromate	53.2	25.9	29.3	46.8
Industrial	69.8	37.0	67.3	36.7
n		8		8
Mean		37.5		39.0
Standard Deviation		23.0		19.1

TABLE 20. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	-	-	-
	Effluent	17.4	27.6	32.6
	Receiving water	-	-	37.4
	Average	17.4	27.6	35.0

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the reference toxicant sample type a majority of the results were outside of the test concentration range, so precision estimates were not calculated. For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 21. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1]

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	100%	97.2	0.00	2.78
	Effluent	25%	65.2	26.1	8.70
	Receiving water	25%	90.0	10.0	0.00
Growth NOEC	Reference toxicant	100%	72.2	11.1	16.7
	Effluent	12.5%	70.8	29.2	0.00
	Receiving water	25%	70.0	30.0	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.