United States
Environmental Protection
Agency

Office of Solid Waste and
Emergency Response
Washington DC 20460

EPA/540/P-91/009
January 1991

EPA

Compendium of ERT Toxicity Testing Procedures

EPA/540/P-91/009
OSWER Directive 9360.4-08
January 1991

COMPENDIUM OF ERT TOXICITY TESTING PROCEDURES

7-Day Standard Reference Toxicity Test using Larval *Pimephales Promelas*

24-Hour Rangefinding Test using *Daphnia Magna* or *Daphnia Pulex*

96-Hour Acute Toxicity Test using Larval *Pimephales Promelas*

24-Hour Rangefinding Test using Larval *Pimephales Promelas*

48-Hour Acute Toxicity Test using *Daphnia Magna* or *Daphnia Pulex*

7-Day Static Renewal Toxicity Test using *Ceriodaphnia Dubia*

7-Day Static Toxicity Test using Larval *Pimephales Promelas*

96-Hour Static Toxicity Test using *Selenastrum Capricornutum*

10-Day Chronic Toxicity Test using *Daphnia Magna* or *Daphnia Pulex*

Interim Final

Environmental Response Team
Emergency Response Division

Office of Emergency and Remedial Response
U.S. Environmental Protection Agency
Washington, DC 20460

Printed on Recycled Paper

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Questions, comments, and recommendations are welcomed regarding the Compendium of ERT Toxicity Testing Procedures. Send remarks to:

Mr. William A. Coakley
Removal Program QA Coordinator
U.S. EPA - ERT
Raritan Depot - Building 18, MS-101
2890 Woodbridge Avenue
Edison, NJ 08837-3679

For additional copies of the Compendium of ERT Toxicity Testing Procedures, please contact:

National Technical Information Service (NTIS)
U.S. Department of Commerce
5285 Port Royal Road
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(703) 487-4600

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Acknowledgments

Preparation of this document was directed by William A. Coakley, the Removal Program QA Coordinator of the Environmental Response Team, Emergency Response Division. Additional support was provided under U.S. EPA contract #68-03-3482 and U.S. EPA contract #68-W0-0036.

1.0 7-DAY STANDARD REFERENCE TOXICITY TEST USING LARVAL *PIMEPHALES PROMELAS*: SOP #2020

1.1 SCOPE AND APPLICATION

The procedure for conducting a standard reference toxicity test using sodium pentachlorophenate (NaPCP) as the toxicant and larval *Pimephales promelas* (fathead minnows) as the test organism is described below. This test estimates the fitness, condition, and sensitivity of the organisms used in a definitive toxicity test. It allows for inter- and intra-laboratory comparisons of toxicity information and provides an experimental control (Lee, 1980). Response of the organisms should be within two standard deviations from the accepted mortality values for the definitive test data to be considered valid (American Public Health Association, 1985). Other standard reference toxicants may be used if justified and the appropriate reference cited. Reference toxicants are available from the U.S. EPA Environmental Monitoring and Support Laboratory, Cincinnati, Ohio.

1.2 METHOD SUMMARY

Fathead minnow larva are exposed to several concentrations of the standard reference toxicant. This test is conducted following the same procedures used for the definitive test. The range of concentrations used in the standard reference toxicant test are selected to encompass the EC_{50} of the standard reference toxicant used. The lethal threshold of NaPCP is 0.1-0.2 mg/L at about 24 hours (Adelman et al., 1980). The U.S. EPA LC_{50} of NaPCP is 0.08-0.19 mg/L.

1.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

1.4 INTERFERENCES AND POTENTIAL PROBLEMS

- When conducting a toxicity test with NaPCP, the pH needs to be kept above 7.4. The toxicity of NaPCP increases as the pH drops, which could give erroneous results (Lee, 1980).
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms, giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

1.5 EQUIPMENT/APPARATUS

1.5.1 Apparatus

- 12 small cups -- glass or plastic
- 12 exposure chambers -- glass or plastic, 2 liters
- 3 graduated cylinders -- 1 liter
- 6 beakers -- 250 mL or a larger volumetric flask -- 2 liters
- 2 mixing buckets or beakers
- pipettes -- 10 mL or smaller

- plastic tubing -- 3/8-inch outside diameter
- plastic screening -- a mesh smaller than the fish
- dilution water -- 11 L/day standard reference toxicant -- NaPCP
- wide-bore pipettes -- 1.5 times the length of the fish
- suitable food

1.5.2 Test Organisms

Test organisms may be reared in-house or received from an outside source. All fathead minnow larva must be less than 24-hours old. To ensure larva less than 24-hours old, use eggs that were laid approximately 3 to 4 days prior to the beginning of the test. Place the substrate containing the eggs into a bucket containing dilution water. This allows the test organisms to become acclimated to the dilution water, reducing stress. Aerate the eggs vigorously to avoid fungal growth and use populations of fish that have less than 5% mortality (American Public Health Association, 1985). Peltier and Weber (1985) and Denny (1987) provide more detailed information, including culturing, caring for, handling, and preventing disease in fathead minnows.

1.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure and record alkalinity and hardness according to a standard method (American Public Health Association, 1985).

1.6 REAGENTS

1.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water.

1.6.2 Test Medium

As a quality control measure, the accuracy of the dilutions should be measured on test concentrations so that results from one test are comparable to other tests. If the reference toxicant is from the U.S. EPA, instructions are included on how to prepare a stock solution. If not, a stock solution should be prepared in advance to facilitate the preparation of test concentrations.

1.7 PROCEDURES

1. Choose a range of concentrations that span those causing zero mortality to those causing complete mortality (indicated by a total absence of movement, even when prodded). Two replicates per concentration and two control replicates following a geometric or logarithmic concentration should be used. Table 1: Example 1 below provides standard reference concentrations that may be used.

2. Label clean exposure chambers, rinse them in dilution water, and then place chambers on a table that will meet test requirements in Table 2. Dilution water must be 25°C ± 2°C.

3. Pour 1 liter of dilution water into each control exposure chamber. Then prepare the NaPCP stock solution by diluting 10 mL of NaPCP up to 100 mL. This will provide a 321 mg/L stock solution.

4. To prepare the first exposure chamber as per Example 1, measure 0.2 mL of the stock solution into a flask and dilute to 2 liters with the dilution water. Pour 1 liter each into the replicate exposure chambers that are labelled 0.03 mg/L.

5. Working in order of increasing concentration, prepare the remaining exposure solutions.

6. After all exposure chambers are filled, the fish may be added to the chambers. Using a wide-bore pipette, select one fish at a time from the test population and place into a small cup. Prepare 12 cups containing 10 fish each.

7. After the 12 cups have been prepared, randomly select a cup for each exposure chamber. Gently submerge the cup below the surface of the dilution and gently pour the fish into the chamber.

8. The addition of the fish signifies the beginning of the test. Record the start time on a data sheet.

9. Measure temperature, pH, conductivity, and dissolved oxygen directly from the exposure chamber and measure hardness and alkalinity from an aliquot removed from a chamber. Measurement should be conducted after the fish have been added to the chambers.

10. Note mortality 2 hours after initiation of the test, and thereafter on a daily basis.

11. Feed larval fish three times daily at 4-hour intervals (e.g., 0800, 1200, and 1600). Use a commercially prepared food suitable to larval fish, or a freshwater-rinsed concentrated suspension of newly-hatched brine shrimp. If brine shrimp are used for food, add approximately 700-1000 nauplii (0.1 mL) to each chamber.

12. New exposure solutions must be prepared daily. Draw out the old exposure solution, waste debris and food as carefully as possible. (Leave sufficient volume to cover the test fish.)

13. Carefully pour the new solution down the sides of the test chamber.

14. Steps 9-12 must be conducted every day of the test.

15. On the last day of the test, renewal of the test solution is not conducted. Live test fish are removed, preserved in 4% buffered formalin, and weighed and measured as required.

1.8 CALCULATIONS

The methods used to determine the EC_{50} differ depending on the results of the test. If there are no partial effects in any replicate (i.e. all alive and healthy or all dead), then the Moving-Average Method may be used to determine the EC_{50}. If there are partial effects within a replicate, then the Probit Method should be used to calculate the EC_{50}. Also the Lowest Observable Effect Concentration (LOEC), the No Observable Effects Concentration (NOEC) and the chronic value (CHV) are recorded (Peltier and Weber, 1985). Measure growth in larva to determine the effect of the standard reference toxicant on the life cycle. Compare the dry weight of the fish in the various concentrations to the dry weight of the control group of fish raised under the same conditions.

1.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 2, for adequate QA/QC.

1.10 DATA VALIDATION

The criteria below provide a basis for rejecting the results generated under this toxicity test:

- Greater than 20% control mortality.
- Greater than 20% aberrant mortality.
- Temperature variation greater than 2°C.

Table 1: Example 1

Standard Reference Concentration (mg/L NaPCP)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	2000.0	0
0.03	1999.8	0.2
0.06	1999.6	0.4
0.08	1999.5	0.5
0.16	1999.0	1.0
0.30	1998.0	2.0

- Standard reference toxicant stored more than 72 hours.
- Criteria in Table 2 not met.

1.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety procedures.

TABLE 2: Summary of Test Conditions for a 7-Day Standard Reference Toxicity Test using Larval *Pimephales promelas**

1.	Test type	Static, daily renewal
2.	Temperature	25°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	2-L containers
7.	Test solution volume	1000 mL/replicate
8.	Renewal	Daily
9.	Age of test organisms	Newly-hatched larva (less than 24 hours old)
10.	Number/container	10 per container
11.	Replicates	Minimum of 2
12.	Feeding	Feed 0.1 mL brine shrimp nauplii three times per day in each container
13.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
14.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
15.	Standard reference toxicant concentrations	Minimum of 5 and 1 control
16.	Test duration	7 days
17.	Effects	Survival and growth

* Based on Horning and Weber, 1985

2.0 24-HOUR RANGEFINDING TEST USING *DAPHNIA MAGNA* OR *DAPHNIA PULEX*: SOP #2021

2.1 SCOPE AND APPLICATION

The procedure for conducting a 24-hour rangefinding toxicity test using *Daphnia magna* or *Daphnia pulex* is described below. This test is applicable to leachates, effluents, and liquid phases of sediments. The selection of concentrations to use in a definitive toxicity test are based on the results of the rangefinder.

2.2 METHOD AND SUMMARY

Larval daphnids are placed in individual containers and exposed to a wide range of concentrations of the test medium. No replicates are needed and only a few concentrations (i.e. 0%, 1%, 10% and 100%) are used.

2.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling; and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

2.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal fluctuation in effluent toxicity (Peltier and Weber, 1985). This is a preliminary test which provides an estimate of toxicity and the results are viewed as such.
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

2.5 EQUIPMENT/APPARATUS

2.5.1 Apparatus

- 25 larval daphnids -- acclimated 24 hours to dilution water
- 4 exposure chambers -- 100 mL/chamber rinsed in dilution water
- tray to hold exposure chambers and glass covers
- wide-bore pipettes -- inside diameter 1.5 times the size of a daphnid
- graduated cylinders, 250 mL
- beakers for chemical measurements
- suitable food
- test medium -- 150 mL
- diluent -- 300 mL
- pipette -- 1 mL
- light table -- to assist in counting the organisms

2.5.2 Test Organisms

Test organisms may be reared in-house or obtained from an outside source. Positive identification of the species is required before testing begins. Daphnids must be less than 24-hours old and from the second to the sixth brood of a healthy adult. Populations of healthy daphnids have large individuals, an absence of floaters, an absence of ephippia, and have an absence of parasites. Individuals are dark colored and produce large numbers of young (Biesinger, et al. 1987).

2.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure and record alkalinity and hardness according to a standard method (American Public Health Association, 1985).

2.6 REAGENTS

2.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. The dilution water for a test is the same as the water used to culture daphnids and the water used to acclimate daphnids before the beginning of the test.

2.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a sediment, preliminary filtration and dilutions are required to produce a liquid phase.

2.7 PROCEDURES

1. Choose a wide range of concentrations to estimate the toxicity of the test medium. The concentrations cited in Table 3: Example 2 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973). The example provides enough test medium for three test chambers containing 80 mL each. In addition, 100 mL each of dilution water and test medium are required for chemical analyses.

2. Measure temperature, pH, conductivity, dissolved oxygen, alkalinity and hardness for all test solutions prior to the start of the test.

3. Label clean exposure chambers and rinse in dilution water, except for the chamber containing 100% test medium.

4. To prepare the first test solution, measure 1.0 mL of the test medium into a beaker and dilute to 100 mL with dilution water.

5. Using a graduated cylinder, pour 80 mL into the exposure chamber. Mix the remaining concentrations in the same manner. Always

Table 3: Example 2

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	100	0
1	99	1
10	90	10
100	0	100

work from the lowest concentration to the highest.

6. Using a wide-bore pipette, randomly select a daphnid, place the pipette below the surface of the test solution and gently expel each daphnid individually into an exposure chamber.

7. The test begins when half of the organisms have been placed into exposure chambers. Mortality (indicated by a total absence of movement, even when prodded) should be determined at 1 hour and again at 24 hours.

2.8 CALCULATIONS

The methods used to determine the LC_{50} differ depending on the results of the test. The Moving-Average Method is used to determine the LC_{50} when there is no partial mortality in any replicate (i.e. all alive or all dead). If there is partial mortality, the Probit Method is used to calculate the LC_{50}. The Lowest Observable Effect Concentration (LOEC) is recorded and the No Observable Effect Concentration (NOEC) is recorded (Peltier and Weber, 1985). Since this is a simple acute test, only mortality is recorded. Other methods of estimating the LC_{50} may be used if justified and an accepted reference is cited (Biesinger, et al. 1987).

2.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 4, for adequate QA/QC.

2.10 DATA VALIDATION

The following criteria provide a basis to reject test results:

- Greater than 10% control mortality.
- Greater than 10% aberrant mortality in concentrations.
- Temperature variation greater than 2°C.
- Effluent stored more than 72 hours.
- Criteria in Table 4 not met.

2.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety procedures.

TABLE 4: Summary of Test Conditions for a 24-Hour Rangefinding Test using *Daphnia magna* or *Daphnia pulex**

1.	Test type	Static, 24 hours
2.	Temperature	20.0°C ± 2°C
3.	Light Quality	Ambient laboratory illumination
4.	Light Intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	100-mL containers
7.	Test solution volume	80 mL/replicate
8.	Age of test organisms	Larval daphnids, less than 24 hours old and within 4 hours of each other
9.	Number/container	10 per container
10.	Feeding	Do not feed during the test
11.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
12.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
13.	Effluent/leachate concentrations	3 and 1 control

*Based on Peltier and Weber, 1975

3.0 96-HOUR ACUTE TOXICITY TEST USING LARVAL *PIMEPHALES PROMELAS*: SOP #2022

3.1 SCOPE AND APPLICATION

The procedure for conducting a 96-hour acute toxicity test using larval *Pimephales promelas* (fathead minnows) is described below. This test is applicable to effluents, leachates, and liquid phases of sediment which require an acute toxicity estimate.

3.2 METHOD SUMMARY

Larval fathead minnows are exposed to different concentrations of a test medium over a 96-hour period. Survival results are used to determine the LC_{50} of the test medium. Test concentrations are renewed daily and mortality is the endpoint of the test.

3.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

3.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal changes in effluent toxicity. This method is less sensitive than a flow-through toxicity test and the sensitivity is dependent on the accuracy of the dilutions (Peltier and Weber, 1985).
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

3.5 EQUIPMENT/APPARATUS

3.5.1 Apparatus

- 120 larval fathead minnows -- less than 30 days old
- 12 exposure chambers -- 1 liter, glass or plastic, labeled
- 12 small cups -- 50 mL
- graduated cylinders -- 1 liter and 10 mL
- mixing bucket -- 2 liters or larger
- plastic tubing -- 3/8-inch outside diameter
- plastic screening dilution
- water -- 4 L/day
- test medium -- 2 L/day
- wide-bore pipettes -- inside diameter 1.5 times the length of the organism
- waste containers
- brine shrimp nauplii

3.5.2 Test Organisms

Larval fathead minnows may be cultured in-house or obtained from an outside source. Positive identification of the species must be made prior to beginning the test. Fish to be used for acclimation and toxicity tests must be healthy and have less than 5% mortality. If test medium and dilution water are limited, use smaller test organisms. This will also ensure that the exposure chambers are not over loaded. Fish selected for acclimation need to be similar in size, not more than 1.5 times the length of each other. Larval fathead minnows must be fed during the acclimation period as well as during the test. Brine shrimp nauplii or other suitable larval fish food may be used. Peltier and Weber (1985) and Denny (1987) provide more detail and information including culturing, care, handling, and disease prevention of fathead minnows.

3.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH, and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure and record alkalinity and hardness using a standard method (American Public Health Association, 1985).

3.6 REAGENTS

3.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water.

3.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a liquid phase of a sediment, preliminary filtration and dilutions are required.

3.7 PROCEDURES

1. Choose a range of concentrations that span those causing zero mortality to those causing complete mortality (indicated by a total absence of movement, even when prodded). The concentrations cited in Table 5: Example 3 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations may also be used (Sprague, 1973). The example below provides six concentrations with two 500-mL replicates.

2. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water. Label the outside of the chambers.

3. Measure 500 mL of dilution water and pour into each control exposure chamber replicate. Then, prepare test concentrations, working from the lowest concentration to the highest.

4. Measure 10 mL of the test medium into a

Table 5: Example 3

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	1000	0
1	990	10
10	900	100
25	750	250
50	500	500
100	0	1000

beaker and dilute to 1000 mL with dilution water. Using a graduated cylinder, pour 500 mL into the two exposure chambers, labelled for 1% test concentration.

5. Repeat step 4 for all concentrations.

6. Using a pipette, randomly place one fish at a time into a small cup until there are 10 fish in each cup. Randomly select the cups and carefully pour the fish into the exposure chambers. Submerge the cup below the test medium surface, gently tilt the cup and pour the fish into the exposure chamber.

7. Record survival at 1 hour and then daily thereafter. Measure and record temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness for all test solutions after addition of the fish.

8. Feed fish during the acclimation period and during the toxicity test. Feed larval fish three times daily at 4-hour intervals (e.g., 0800, 1200, and 1600). Use a freshwater-rinsed, concentrated suspension of newly-hatched brine shrimp. Add approximately 700-1000 nauplii (0.1 mL) to each container (Horning and Weber, 1985). Other food may be used if it is suitable larval fish food.

9. Test solutions must be replaced daily. Using a length of plastic tubing covered with netting, siphon out the concentrations from the exposure chambers. Leave a small amount of test solution in the bottom of the chamber. While siphoning, remove as much dead brine shrimp and waste debris as possible. Mix concentrations as done the day before and slowly pour the new concentrations into the exposure chambers. The temperatures of the new test concentrations must be equal to the temperature of the exposure chamber so that the fish are not stressed.

10. The test is complete after the 96-hour final mortality and chemical measurements are recorded. Dispose of test solution in a manner consistent with good lab practices.

3.8 CALCULATIONS

The methods used to determine the LC_{50} differ depending on the results of the test. If there is no partial mortality in any replicate (i.e. all alive or all dead), then the Moving-Average Method may be used to determine the LC_{50}. If there is partial mortality within a replicate, then the Probit Method should be used to calculate the LC_{50}. Also the Lowest Observable Effect Concentration (LOEC) is recorded and the No Observable Effects Concentration (NOEC) is recorded (Peltier and Weber, 1985). Other methods may be used if justified and the appropriate reference cited. See Sprague (1973) or Peltier and Weber (1985) for more detail on the calculations.

3.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 6, for adequate QA/QC.

3.10 DATA VALIDATION

The following criteria provide a basis for rejecting the results of this test:

- Greater than 10% control mortality.
- Greater than 10% aberrant mortality.
- Temperature variation greater than 2°C.
- Test medium stored more than 72 hours.
- Criteria in Table 6 not met.

3.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA, and specific health and safety procedures.

Table 6: Summary of Conditions for a 96-Hour Toxicity Test using *Pimephales promelas**

1.	Test type	Static, daily renewal
2.	Temperature	25.0°C ± 2°C
3.	Light Quality	Ambient laboratory illumination
4.	Light Intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	1-L container
7.	Test solution volume	500 mL/replicate
8.	Renewal	Daily
9.	Age of test organisms	Less than 30 days old
10.	Number/container	10 per container
11.	Replicates	Minimum of 2
12.	Feeding	Feed 3 times daily
13.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
14.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
15.	Test media concentrations	Minimum of 5 and 1 control
16.	Test duration	96 hours

* Based on Peltier and Weber, 1985.

4.0 24-HOUR RANGEFINDING TEST USING LARVAL *PIMEPHALES PROMELAS*: SOP #2023

4.1 SCOPE AND APPLICATION

The procedure for conducting a 24-hour rangefinding test using larval *Pimephales promelas* (fathead minnows) is described below. This test is used as a preliminary guide when testing an effluent, leachate, or liquid phase of a sediment with an unknown toxicity. The results of this test are used to determine the concentration range in a definitive toxicity test.

4.2 METHOD SUMMARY

Larval fathead minnows are exposed to various concentrations of a test medium over a 24-hour period. Survival and mortality data are used to determine the concentration range to be used in a static or flow-through toxicity test.

4.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and other procedures applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

4.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal changes in effluent toxicity (Peltier and Weber, 1985). This method is less sensitive than a flow-through toxicity test and the sensitivity is dependent on the accuracy of the dilutions.
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

4.5 EQUIPMENT/APPARATUS

4.5.1 Apparatus

- 40 fathead minnows -- less than 30 days old
- 4 small cups -- 50 mL
- 4 exposure chambers -- 1 liter, glass or plastic, labeled
- graduated cylinders -- 1 liter and 10 mL
- mixing bucket -- 1 liter or larger
- plastic tubing -- 3/8" outside diameter
- plastic screening
- dilution water -- 3 liters
- test medium -- 1.5 liters
- wide-bore pipettes -- inside diameter 1.5 times the length of the organism
- waste containers
- brine shrimp or other suitable food

4.5.2 Test Organisms

Test organisms may be reared in-house or received from an outside source. Positive identification of the test organisms must be made prior to starting

the test. The fish to be used for a rangefinding test must be the same age (less than 30 days old), in the same condition, and come from the same culture as those to be used for the definitive test. Place fish into a holding tank and slowly drip the dilution water into the tank over a 24-hour period. Then leave the fish in this water for another 24 hours so that the fish become acclimated to the dilution water. Use populations of fish that are healthy and have less than 5% mortality. For more detailed information, including culturing, caring for, handling, and disease prevention of *Pimephales promelas*, see Peltier and Weber (1985) and Denny (1987).

4.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Use a standard method to measure alkalinity and hardness (American Public Health Association, 1985). Record all measurements on data sheets.

4.6 REAGENTS

4.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. The dilution water for a test is the same as the water used to acclimate the fish before the beginning of the test.

4.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a liquid phase of a soil, preliminary filtration and dilutions are required.

4.7 PROCEDURES

1. In order to determine the range of concentrations to be used for a definitive toxicity test, a preliminary rangefinding test is conducted. Ten fish are placed into exposure chambers with a broad range of concentrations (0%, 1%, 10%, and 100% test media).

2. Survival and mortality (indicated by a total absence of movement, even when prodded) are recorded after 1 hour and 24 hours and the results are used to determine definitive test concentrations.

3. Replicates are not necessary for this test.

4. The concentrations cited in Table 7: Example 4 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973). Other ranges may be used according to the needs of the specific situation.

5. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water.

6. Measure 750 mL of dilution water and pour into the control exposure chamber.

Table 7: Example 4

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	750	0.0
1	742.5	7.5
10	675.0	75.0
100	0.0	750.0

7. Measure 7.5 mL of test medium and dilute to 750 mL with dilution water. Pour this mixture into the exposure chamber. Continue this procedure until all the concentrations are prepared. Always go from the lowest concentration to the highest in order to minimize the risk of cross-contamination.

8. Using a wide bore pipette, randomly put one fish at a time into a small cup, placing 10 fish into each cup. After all the fish have been selected, pour into the exposure chambers by gently submerging the cup below the water surface and pouring the fish out.

9. Measure and record temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness for each test solution after the fish have been added to the exposure chamber, which constitutes the beginning of the test.

4.8 CALCULATIONS

The methods used to determine the LC_{50} differ depending on the results of the test. If there is no partial mortality in any replicate (i.e. all alive or all dead), then the Moving-Average Method may be used to determine the LC_{50}. If there is partial mortality within a replicate, then the Probit Method should be used to calculate the LC_{50} (Peltier and Weber, 1985). Since the results of this test are only preliminary, exact calculations need not be made. An estimate of the LC_{50} is needed to determine the range of concentrations to be used for the definitive test.

Other methods to determine the LC_{50} of the test medium may be used if justified and the appropriate reference cited.

4.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 8, for adequate QA/QC.

4.10 DATA VALIDATION

The following criteria provide a basis for rejecting the results generated under this test:

- Greater than 10% control mortality.
- Criteria in Table 8 not met.

Note: Since this is only a preliminary test, the strict guidelines used for the definitive test need not be adhered to. However, this test should be run according to standard laboratory guidelines.

4.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA, and specific health and safety procedures.

Table 8: Summary of Test Conditions for a 24-Hour Rangefinding Toxicity Test using *Pimephales promelas**

1.	Test type	Static
2.	Temperature	25.0°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	1-L containers
7.	Test solution volume	750 mL
8.	Renewal	None
9.	Age of test organisms	Newly hatched larva (less than 24 hours old)
10.	Number/container	10 per chamber
11.	Feeding	None
12.	Washing	N/A
13.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
14.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
15.	Test media/leachate concentrations	Minimum of 3 and 1 control
16.	Test duration	24 hours

* Based on Peltier and Weber, 1985.

5.0 48-HOUR ACUTE TOXICITY TEST USING *DAPHNIA MAGNA* OR *DAPHNIA PULEX*: SOP #2024

5.1 SCOPE AND APPLICATION

The procedure for conducting a 48-hour acute toxicity test using *Daphnia magna* or *Daphnia pulex* is described below. This test is applicable to leachates, effluents, and liquid phases of sediments.

5.2 METHOD SUMMARY

Larval daphnids are placed in individual containers and exposed to various concentrations of a test medium over a 48-hour period. Mortality is the endpoint of the test.

5.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

5.4 INTERFERENCES AND POTENTIAL PROBLEMS

- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms, giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).
- The results of a static toxicity test do not reflect temporal fluctuation in test media toxicity (Peltier and Weber, 1985).

5.5 EQUIPMENT/APPARATUS

5.5.1 Apparatus

- 60 larval daphnids -- acclimated for at least 24 hours to dilution water
- 60 exposure chambers -- 100 mL volume, labeled
- tray to hold exposure chambers and glass covers
- wide-bore pipettes -- inside diameter 1.5 times the length of the daphnid
- graduated cylinders, 250 mL and 1 liter
- pipette -- 1 mL
- beakers for chemical measurements, 250 mL
- test medium -- 1 liter
- diluent -- 3 liters
- waste containers
- light table -- to aid in counting the organisms
- suitable food

5.5.2 Test Organisms

Test organisms may be reared in-house or obtained from an outside source. Positive identification of the species is required before beginning testing. Daphnids to be used must be less than 24 hours old and from the second to the sixth brood of healthy adults. Populations of healthy daphnids have large individuals, have an absence of floaters, have an absence of ephippia, and have an absence of parasites. Individuals are dark colored and produce

large numbers of young (Biesinger, et al. 1987).

5.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure alkalinity and hardness according to a standard method (American Public Health Association, 1985).

5.6 REAGENTS

5.6.1 Dilution Water

Dilution water is reconstituted, deionized water. The water type should be moderately hard unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. The dilution water for a test is the same as the water used to culture daphnids and the water used to acclimate daphnids before the beginning of the test.

5.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a sediment, preliminary filtration and dilutions are required to produce a liquid phase.

5.7 PROCEDURES

1. Select a range of concentrations that span those causing zero mortality to those causing complete mortality (indicated by a total absence of movement, even when prodded). The concentrations cited in Table 9: Example 5 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973). The example provides enough test medium for five replicates containing 50 mL each and extra for chemical analysis.

2. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water. Label all chambers.

3. Mix concentrations and pour into each exposure chamber. Work from the lowest concentration to the highest in order to minimize the risk of cross-contamination.

4. Measure 0.5 mL of the test medium into a beaker and dilute to 500 mL.

5. Using a graduated cylinder, pour out 50 mL into each exposure chamber which is labeled for 0.1% test concentration. Pour the rest into a beaker for chemical measurements.

6. Repeat steps 4 and 5 for all concentrations.

7. Using a wide-bore pipette, randomly select and carefully place 10 daphnids into each exposure

Table 9: Example 5

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0.0	500	0.0
0.1	499.5	0.5
1.0	495	5.0
10	450	50
50	250	250
100	0	500

chamber by placing the pipette tip below the surface and gently expelling each daphnid individually into the chamber.

8. The test begins when half of the organisms are in the exposure chambers.

9. Measure and record mortality and survival at one hour and then at 24 and 48 hours.

10. Measure and record temperature, dissolved oxygen, pH, conductivity, alkalinity, and hardness for all test solutions after the test begins and at the completion of the test.

11. The test is complete at the end of 48 hours.

5.8 CALCULATIONS

The methods used to determine the LC_{50} differ depending on the results of the test. If there is no partial mortality in any replicate (i.e. all alive or all dead), then the Moving-Average Method may be used to determine the LC_{50}. If there is partial mortality within a replicate, then the Probit Method should be used to calculate the LC_{50}. Also the Lowest Observable Effect Concentration (LOEC) is recorded and the No Observable Effects Concentration (NOEC) is recorded (Peltier and Weber 1985). Since this is a simple acute test, only mortality is recorded. Other methods of estimating the LC_{50} may be used if justified and an accepted reference is cited (Biesinger, et al. 1987).

5.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 10, for adequate QA/QC.

5.10 DATA VALIDATION

The following criteria provide a basis for rejecting the results of this test:

- Greater than 10% control mortality.
- Greater than 10% aberrant mortality in concentrations throughout the test range. However, there may be greater than 10% mortality in one replicate if there is 100% survival above that value.
- Temperature variation greater than 2°C.
- Test medium stored more than 72 hours.
- Criteria in Table 10 not met.

5.11 HEALTH AND SAFETY

When working with potentially hazardous materials, refer to U.S. EPA, OSHA, and specific health and safety procedures.

Table 10: Summary of Test Conditions for a 48-Hour Acute Toxicity Test using *Daphnia magna* or *Daphnia pulex**

1.	Test type	Static, daily renewal
2.	Temperature	20.0°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	100-mL containers
7.	Test solution volume	50 mL/replicate
8.	Renewal	None
9.	Age of test organisms	Less than 24 hours old
10.	Number/container	10 per exposure chamber
11.	Feeding	Do not feed during test
12.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
13.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
14.	Test media/leachate concentrations	Minimum of 5 and 1 control
15.	Test duration	48 hours
16.	Effects measured	Survival at 1, 24, and 48 hours

* Based on Peltier and Weber, 1985

6.0 7-DAY STATIC RENEWAL TOXICITY TEST USING *CERIODAPHNIA DUBIA*: SOP #2025

6.1 SCOPE AND APPLICATION

The procedure for conducting a 7-day static renewal toxicity test using *Ceriodaphnia dubia* is described below. This test is applicable to effluents, leachates, and liquid phases of sediments which require a chronic toxicity estimate. This method uses reproductive success as well as mortality as end points for the test.

6.2 METHOD SUMMARY

Ceriodaphnia dubia are placed in individual exposure chambers containing 15 mL of the test medium concentration. Mortality and survival are recorded over a 7-day period as well as the number of broods, the brood size, and live or dead young. These data are used to determine the Lowest Observable Effect Concentration (LOEC), the No Observable Effect Concentration (NOEC), the EC_{50} and the chronic value of the test medium.

6.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

6.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal changes in effluent toxicity (Peltier and Weber, 1985). This method is less sensitive than a flow-through toxicity test and the sensitivity is dependent on the accuracy of the dilutions.
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

6.5 EQUIPMENT/APPARATUS

6.5.1 Apparatus

- 75 *Ceriodaphnia dubia* -- less than 24 hours old and released during the same 4-hour period
- 60 exposure chambers/day -- 30 mL or larger, labeled
- trays and glass covers for exposure chambers
- wide-bore pipettes -- inside diameter 1.5 times the length of the organisms
- dilution water -- 1.5 L/day
- test medium -- 500 mL/day
- graduated cylinder -- 500 mL and 10 mL
- mixing bucket -- 500 mL or larger
- pipettes -- 1 mL and bulb
- beakers -- 250 mL
- light table -- to aid in counting the organisms
- suitable food
- waste containers

6.5.2 Test Organisms

Test organisms may be reared in-house or obtained from an outside source. Positive identification of *Ceriodaphnia dubia* is required before beginning the test (Berner, 1986). *Ceriodaphnia dubia* to be used must be less than 24-hours old and from the second to the sixth brood of an healthy adult. Adults to be used should be placed into individual cups containing dilution water 24 hours prior to the start of the test in order to ensure less than 24-hour old organisms.

6.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Use a standard method to measure and record alkalinity and hardness (American Public Health Association, 1985). Record all measurements on data sheets.

6.6 REAGENTS

6.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. The dilution water used in a test should be the same as the water used to culture and acclimate the test species.

6.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a sediment, preliminary filtration and dilutions are required to produce a liquid phase.

6.7 PROCEDURES

1. Select a range of concentrations that span those causing zero mortality to those causing complete mortality (indicated by a total absence of movement, even when prodded). The concentrations cited in Table 11: Example 6 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973). The example provides enough effluent for 10 exposure chambers per concentration, each containing 15 mL and extra for chemical analysis. Other ranges may be used according to needs of the analyses.

2. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water.

3. To prepare the first test solution, measure 0.30 mL of the test medium into a beaker and dilute to 300 mL using dilution water.

4. Using a graduated cylinder, pour 15 mL into each exposure chamber labeled for .1% test

Table 11: Example 6

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0.0	300	0.0
0.1	299.7	0.3
1.0	297	3.0
10	270	30
50	150	150
100	0	300

concentration and pour the rest into a beaker for chemical analyses.

5. Continue steps 3 and 4 for all concentrations. Always work from lowest concentration to the highest in order to minimize the risk of cross-contamination.

6. Using a wide-bore pipette, randomly select one acclimated *Ceriodaphnia dubia* (under 24-hours old) into each cup by placing the organism under the surface of the test medium and gently expelling it into the test chamber.

7. Add 0.1 mL (1 drop) of a suitable food to each exposure chamber as food.

8. Measure and record survival at 1 hour.

9. Measure and record temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness daily of all new test solutions.

10. Measure and record dissolved oxygen daily from both old and new test solutions and the control. Do this prior to pouring the test concentrations into the individual exposure chambers.

11. On the second day, prepare new test medium concentrations and a new set of exposure chambers.

12. Pour new concentrations into new chambers as done previously and use the excess for chemical analyses.

13. Count the number of broods, the brood size, and the number of live or dead organisms. *Ceriodaphnia dubia* usually start to produce offspring after the third day of the test and they should have three broods by the completion of the test. The endpoint of the test is when 60% of the control organisms have at least three broods and at least 90 young (an average of nine per organism).

14. Place 0.1 mL (1 drop) of a suitable food into the exposure chambers after the concentrations have been renewed but before the test organisms are transferred into the chamber. This provides for more consistent water quality between changes.

15. Transfer adult *Ceriodaphnia dubia* by carefully removing with a wide-bore pipette and transferring into the new exposure chamber.

16. Place a cover loosely over the exposure chambers to prevent evaporation.

6.8 CALCULATIONS

The methods used to determine the EC_{50} differ depending on the results of the test. If there are no partial effects in any replicate (i.e. all alive and healthy or all dead), then the Moving-Average Method may be used to determine the EC_{50}. If there are partial effects within a replicate, then the Probit Method should be used to calculate the EC_{50}. Also the Lowest Observable Effect Concentration (LOEC), the No Observable Effects Concentration (NOEC) and the chronic value are recorded (Peltier and Weber, 1985). Other methods of determining the EC_{50} may be used if justified and the appropriate reference is cited.

6.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 12, for adequate QA/QC.

6.10 DATA VALIDATION

The following criteria provide a basis for rejecting the results of this test:

- Greater than 20% control mortality.
- Greater than 20% aberrant mortality in any concentrations.
- Temperature variation greater than 2°C.
- Test medium stored more than 72 hours.
- Criteria in Table 12 not met.
- Less than 3 broods in the control group and less than 90 young produced in the control group (an average of 9 per individual).

6.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety procedures.

Table 12: Summary of Test Conditions for 7-Day Static Renewal Toxicity Test using *Ceriodaphnia dubia**

1.	Test type	Static, daily renewal
2.	Temperature	25.0°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	30-mL containers
7.	Test solution volume	15 mL per exposure chamber
8.	Renewal	Daily
9.	Age of test organisms	Newly hatched larva (less than 24 hours old)
10.	Number/container	1 per chamber (10 chambers)
11.	Feeding	Feed 1 drop (0.1 mL) of suitable food per day
12.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
13.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
14.	Test media/leachate concentrations	Minimum of 5 and 1 control
15.	Test duration	7 days
16.	Effects measured	Survival and reproduction

* Based on Horning and Weber, 1985.

7.0 7-DAY STATIC RENEWAL TOXICITY TEST USING LARVAL *PIMEPHALES PROMELAS*: SOP #2026

7.1 SCOPE AND APPLICATION

The procedure for conducting a 7-day static renewal toxicity test using larval *Pimephales promelas* (fathead minnows) is described below. This test is applicable to effluents, leachates, and sediments which require a chronic toxicity estimate.

7.2 METHOD SUMMARY

Larval fathead minnows are exposed to different concentrations of a test medium over a 7-day period. Survival and growth results are used to determine the No Observable Effect Concentration (NOEC), the Lowest Observable Effect Concentration (LOEC), the EC_{50}, and the chronic value (CHV) of the test medium. Test concentrations are renewed daily.

7.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

7.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal changes in effluent toxicity. This method is less sensitive than a flow-through toxicity test and the sensitivity is dependent on the accuracy of the solutions (Peltier and Weber, 1985).
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms, giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).

7.5 EQUIPMENT/APPARATUS

7.5.1 Apparatus

- 120 larval fathead minnows -- less than 24 hours old
- 12 exposure chambers -- 1 liter, labeled
- 12 small cups -- 50 mL
- test medium -- 2 L/day
- diluent -- 4.25 L/day
- graduated cylinders -- 3, 1 liter
- beakers -- 250 mL
- mixing buckets -- 2 liters
- plastic tubing -- 3/8-inch outside diameter
- plastic screening -- mesh with smaller than that of the fish
- wide-bore pipettes -- inside diameter 1.5 times the size of the fish
- waste containers
- brine shrimp or other suitable food

7.5.2 Test Organisms

Larval fathead minnows may be cultured in-house or obtained from an outside source. Positive identification of the species must be made prior to beginning the test. Fathead minnows to be used for the test must be healthy. Place the substrate holding the eggs into the dilution water 24 hours prior to the beginning of the test to ensure that the fish to be used are less than 24-hours old. Larval fathead minnows must be fed during the acclimation period as well as during the test. Brine shrimp nauplii or other suitable larval fish food may be used. Peltier and Weber (1985) and Denny (1987) provide more detailed information, including culturing, caring for, handling, and preventing disease in fathead minnows.

7.5.3 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure and record alkalinity and hardness using a standard method (American Public Health Association, 1985).

7.6 REAGENTS

7.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. Set up a laboratory or standard dilution water control when receiving waters are used as the dilution water.

7.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is to be a liquid phase of a soil, preliminary filtration and dilutions are required.

7.7 PROCEDURES

1. Choose a range of concentrations that span those causing no effect to those causing complete mortality (indicated by a total absence of movement, even when prodded). The concentrations cited in Table 13: Example 7 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973). The example provides enough test medium for two replicates containing 500 mL each.

2. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water.

3. Prepare the test dilutions by pouring 500 mL of dilution water into both control chambers. Then measure out 10 mL of the test medium into a bucket and pour 990 mL of dilution

Table 13: Example 7

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	1000	0
1	990	10
10	900	100
25	750	250
50	500	500
100	0	1000

water into the bucket and mix. Using a graduated cylinder, pour 500 mL into both 1% exposure chambers.

4. Repeat step 3 for all concentrations. Always work from the lowest concentration to the highest in order to minimize the risk of cross-contamination.

5. Using a pipette, randomly place one fish at a time into a small cup until there are 10 fish in each cup.

6. Randomly select the cups and carefully pour the fish into the exposure chambers by submerging the cup below the test medium surface, gently tilting the cup and pouring the fish into the exposure chamber.

7. Record survival at 1 hour and then daily thereafter.

8. Measure and record dissolved oxygen, temperature, pH, conductivity, alkalinity and hardness of all test solutions after the fish have been placed into the chambers and then daily thereafter.

9. Feed larval fish three times daily at 4-hour intervals (e.g., 0800, 1200, and 1600). Use a commercially prepared food suitable to larval fish or a freshwater-rinsed concentrated suspension of newly-hatched brine shrimp. If using shrimp, add approximately 700-1000 nauplii (0.1 mL) to each container.

10. Prepare new dilutions daily.

11. Place plastic screening over a length of tubing and create a siphon using the dilution water. Carefully draw out as much of the old solution, dead brine shrimp and waste debris as possible from the exposure chamber without disturbing the fish. Again, work from the lowest concentration to the highest in order to minimize the risk of cross-contamination.

12. Discard tubing and the waste concentrations in a manner consistent with standard laboratory procedures.

13. Carefully pour the new test solutions into the exposure chambers. Steps 10 - 12 are repeated each day except for the last day of the test.

7.8 CALCULATIONS

The methods used to determine the EC_{50} differ depending on the results of the test. If there are no partial effects in any replicate (i.e. all alive and healthy or all dead), then the Moving-Average Method may be used to determine the EC_{50}. If there are partial effects within a replicate, then the Probit Method should be used to calculate the EC_{50}. Also the Lowest Observable Effect Concentration (LOEC), the No Observable Effect Concentration (NOEC) and the chronic value (CHV) are recorded (Peltier and Weber, 1985). Growth is also measured in the larva to determine the effect of the test medium on the life cycle. This is done by comparing the dry weight of the fish in the various concentrations to the dry weight of a control group of fish raised under the same conditions.

7.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 14, for adequate QA/QC.

7.10 DATA VALIDATION

The following criteria provide a basis for rejecting the results of this test:

- Greater than 20% control mortality.
- Greater than 20% aberrant mortality.
- Temperature variation greater than 2°C.
- Test medium stored more than 72 hours.
- Criteria in Table 14 not met.

7.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety procedures.

Table 14: Summary of Test Conditions for 7-Day Static Renewal Toxicity Test using Larval *Pimephales promelas**

1.	Test type	Static, daily renewal
2.	Temperature	25.0°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	1-L containers
7.	Test solution volume	500 mL/replicate
8.	Renewal	Daily
9.	Age of test organisms	Newly hatched larva (less than 24 hours old)
10.	Number/container	10 per container
11.	Replicates	Minimum of 2
12.	Feeding	Feed 0.1 mL of brine shrimp nauplii 3 times per day, in each container
13.	Washing	Siphon daily before solution renewal
14.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
15.	Dilution water	Moderately hard, reconstituted, deionized water, unless otherwise specified
16.	Test media/leachate concentrations	Minimum of 5 and 1 control
17.	Test duration	7 days
18.	Effects measured	Survival and growth (increase in weight)

* Based on Horning and Weber, 1985

8.0 96-HOUR STATIC TOXICITY TEST USING *SELENASTRUM CAPRICORNUTUM*: SOP #2027

8.1 SCOPE AND APPLICATION

The procedure for conducting a 96-hour static toxicity test using *Selenastrum capricornutum* is described below. The endpoint of this test is growth, measured by increase in cell count, chlorophyll content, biomass, or absorbance (turbidity). This test may be conducted on effluents, leachates or liquid phase of sediments. This test will also identify a test medium that is biostimulatory (Horning and Weber, 1985).

8.2 METHOD SUMMARY

Selenastrum capricornutum is exposed to various concentrations of a test medium over a 96-hour period and growth is measured at the end of the test.

8.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

8.4 INTERFERENCES AND POTENTIAL PROBLEMS

- The results of a static toxicity test do not reflect temporal changes in effluent toxicity.
- The detection limits of the toxicity of a test medium are organism dependent (Horning and Weber, 1985).
- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).
- The concentrations of natural nutrients in the test medium may affect the results (Horning and Weber, 1985).

8.5 EQUIPMENT/APPARATUS

8.5.1 Apparatus

- *selenastrum capricornutum* culture
- 18 Erlenmeyer flasks -- 250 mL
- dilution water -- 1.5 liters
- test medium -- 1 liter
- stock nutrient solutions
- centrifuge -- 15 - 100 mL capacity
- graduated cylinders -- 10 mL and 100 mL
- Erlenmeyer flask -- 500 mL
- microscope

Depending on the method used to calculate growth, other equipment may be necessary.

8.5.2 Washing Procedure

1. Wash with warm tap water and non-phosphate detergent.
2. Rinse with tap water.
3. Rinse with 10% HCl.

4. Rinse with deionized water.

5. Rinse with 100% acetone.

6. Rinse with deionized water.

7. Final rinse with dilution water.

8.5.3 Test Organisms

Selenastrum capricornutum may be raised in-house or received from an outside source. Positive identification of the species is required before beginning the test. A stock culture that is 4 to 7 days old is required for this test. Horning and Weber (1985) provide detailed information on the preparation of culture medium and stock culture.

8.5.4 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH, and conductivity. Calibrate the meters according to the manufacturer's specifications. Measure and record alkalinity and hardness according to a standard method (American Public Health Association, 1985).

8.6 REAGENTS

8.6.1 Dilution Water

Dilution water is moderately hard, reconstituted deionized water unless otherwise specified. The dilution water for the test is the same water used to culture *Selenastrum capricornutum*. See Horning and Weber (1985) for the preparation of synthetic fresh water.

8.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a liquid phase of a sediment, preliminary filtration and dilutions are required. To eliminate false negative results due to low nutrient concentrations, add 1 mL of stock culture solution (except EDTA) per liter of test medium prior to preparing test concentrations.

8.6.3 Stock Culture Solution

The methods needed to prepare the stock culture solution and the amount of chemicals needed to prepare the solution are found in Horning and Weber, 1985. One liter of test medium will provide three replicates of 100 mL each for six concentrations and 400 mL for chemical analyses (Horning and Weber, 1985).

Table 15: Example 8

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
0	300	0
1	297	3
3	291	9
10	270	30
30	210	90
100	0	300

8.7 PROCEDURES

1. Maintain a stock culture of algae at 24°C ± 2°C under continuous lighting.

2. Transfer 1 - 2 mL aseptically to new test medium once a week in order to maintain an uncontaminated and healthy culture.

3. To prepare the inoculum, follow the steps below (Horning and Weber, 1985).

4. An inoculum is prepared from the stock solution 2 to 3 hours prior to the beginning of the test. Each milliliter of inoculum must contain enough cells to provide an initial cell density of 10,000 cells/mL in the exposure chamber. Therefore, each milliliter of inoculum must contain 1 million cells if using 100 mL test volume. Use the formula below to determine the amount of stock solution required for the test.

5. Volume of stock solution required (mL) = (# of flasks) (vol. of test soln. per flask) x 10,000 cells/mL cell density in stock culture.

   a. Determine the density of cells in the stock solution.
   b. Calculate the required volume of stock solution (from the equation above).
   c. Centrifuge 50% more than the calculated value of stock solution at 1000 x g (g = gravitational constant) for 5 minutes.
   d. Decant the supernatant and resuspend in 15 mL of deionized water.
   e. Repeat steps c and d.
   f. Mix and determine the cell count and dilute as necessary to obtain a cell density of 106 cells/mL.

6. If possible, choose a range of concentrations that will span those with no effect to that which will cause complete mortality. The concentrations in Table 15: Example 8 may be adjusted to meet the specific needs of the test.

7. Measure 100 mL of dilution water into each of the three control flasks.

8. Mix 3 mL of test medium with 297 mL of dilution water into a mixing bucket.

9. Pour 100 mL into each 1% test medium flask.

10. Continue with these dilutions until all concentrations are mixed.

11. Add 1 mL of test inoculum to each flask and begin the test.

12. At 1 to 2 hours, check the cell density of the controls to ensure sufficient test organisms. There are no renewals of test solutions for the duration of the test and the test is complete at 96 hours.

13. Measure and record temperature, dissolved oxygen, pH, conductivity, alkalinity, and hardness on all test solutions.

14. Growth is measured at the end of the test by cell counts, chlorophyll content or turbidity (light absorbance), or biomass. Cell counts may be determined with an automatic particle counter or manually under a microscope. Chlorophyll content may be measured using in-vivo or in-vitro fluorescence or in-vitro spectrophotometry. Turbidity may be measured by spectrophotometry at 750 nm. Biomass is measured by multiplying the cell count by the mean cell volume or by direct gravimetric dry weight analysis. Horning and Weber (1985) provide details of the methodologies for these measurements.

15. At the completion of the test, samples should be checked under a microscope to detect any abnormal cell growth or other deviations.

16. It also may be necessary to check algal growth on a daily basis depending on the test medium.

8.8 CALCULATIONS

The No Observable Effect Concentration (NOEC), the Lowest Observable Effect Concentration (LOEC), and the chronic value (CHV) are measured and recorded at the end of 96 hours. Dunnetts procedure or the Probit Method may be used to calculate the NOEC and LOEC. When the assumptions for normality and homogeneity of variance are not met, Steel's Many - One Rank Test may be used. Other methods may be used if justified and the appropriate method is cited. Calculate the percent stimulation (%S) if growth in the concentrations exceeds the growth in the controls.

8.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 16, for adequate QA/QC.

8.10 DATA VALIDATION

Test data is invalidated for the following reasons.

- Cell density in the controls is less than 106 cell/mL at the end of the test and the number does not vary by more than 10% between control replicates.
- Parameters in Table 16 are not met.

8.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety guidelines.

Table 16: Summary of Test Conditions for a 96-Hour Static Toxicity Test using *Selenastrum capricornutum**

1.	Test type	Static, non-renewal
2.	Temperature	25.0°C ± 2°C
3.	Light intensity	400 ± 40 foot candles
4.	Photoperiod	Continuous
5.	Exposure chamber size	250-mL containers
6.	Test volume	100 mL
7.	Stock culture	4-7 days old
8.	Cell density	10,000 cells per mL
9.	Replicates	3 per concentration
10.	Shaking rate	Twice daily by hand or 100 cpm
11.	Dilution water	Reconstituted, deionized water, unless otherwise specified. Also the same as the culture water without the EDTA
12.	Test duration	96 hours
13.	Effects measured	Growth

* Based on Horning and Weber, 1985

9.0 10-DAY CHRONIC TOXICITY TEST USING *DAPHNIA MAGNA* OR *DAPHNIA PULEX*: SOP #2028

9.1 SCOPE AND APPLICATION

The procedure for conducting a 10-day chronic toxicity test using *Daphnia magna* or *Daphnia pulex* is described below. This test is applicable to leachates, effluents, and liquid phases of sediments. Mortality, reproduction and growth are used to assess the toxicity of the test medium.

9.2 METHOD SUMMARY

Larval daphnids are placed in individual containers and exposed to different concentrations of a test medium over a 10-day period. Concentrations are renewed every other day and mortality, reproduction and growth are recorded.

9.3 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental medium will be sampled utilizing the methodology detailed in ERT Standard Operating Procedures (SOPs) #2012, Soil Sampling; #2013, Surface Water Sampling; #2016, Sediment Sampling, and any other procedure applicable to the medium sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because surrogate test species will be exposed to varying concentrations of the sample material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice at 4°C for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

9.4 INTERFERENCES AND POTENTIAL PROBLEMS

- Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the organisms giving false results.
- Dissolved oxygen depletion due to biological oxygen demand and/or chemical oxygen demand (e.g., metabolic wastes) is also a potential problem.
- Loss of a toxicant through adsorption to exposure chambers and volatilization may occur (Peltier and Weber, 1985).
- The results of a static toxicity test do not reflect temporal fluctuation in test medium toxicity (Peltier and Weber, 1985). Also the effect of the toxicant is organism dependent.

9.5 EQUIPMENT/APPARATUS

9.5.1 Apparatus

- 60 larval daphnids -- acclimated at least 24 hours to dilution water
- 60 exposure chambers -- 100 mL volume, labeled
- tray to hold exposure chambers and glass covers
- wide-bore pipettes -- inside diameter 1.5 times the length of the daphnid
- graduated cylinders -- 250 mL and 1 liter
- pipette -- 1 mL
- beakers -- 250 mL
- volumetric flasks -- 500 mL
- test medium -- 1 L/day
- diluent -- 3 L/day
- waste containers
- light table -- to assist in counting the organisms
- suitable food

9.5.2 Washing Procedure

1. Wash with warm water and detergent
2. Rinse with tap water

3. Rinse with 10% nitric acid solution

4. Rinse with deionized water

5. Rinse with 100% acetone

6. Rinse with deionized water

7. Final rinse with dilution water.

9.5.3 Test Organisms

Test organisms may be reared in-house or obtained from an outside source. Positive identification of the species is required before beginning the test. Daphnids to be used must be less than 24 hours old and from the second to the sixth brood of a healthy adult. Populations of healthy daphnids have large individuals, have an absence of floaters, have an absence of ephippia, and have an absence of parasites. Individuals are dark colored and produce large numbers of young (Biesinger, et al. 1987).

9.5.4 Equipment for Chemical Analysis

Meters are needed to measure dissolved oxygen, temperature, pH and conductivity. Calibrate the meters according to the manufacturer's instructions. Measure alkalinity and hardness according to a standard method (American Public Health Association, 1985).

9.6 REAGENTS

9.6.1 Dilution Water

Dilution water is reconstituted deionized water unless otherwise specified. See Horning and Weber (1985) for the preparation of synthetic fresh water. Set up a laboratory or standard dilution water control when reconstituted deionized water is used as the dilution water. The dilution water for a test is the same as the water used to culture daphnids and the water used to acclimate daphnids before the beginning of the test.

9.6.2 Test Medium

If the test medium is a liquid, dilutions may be made directly for the required concentrations. If the test medium is a sediment, preliminary filtration and dilutions are required to produce a liquid phase.

9.7 PROCEDURES

1. Choose a range of concentrations that span those causing zero mortality to those causing complete mortality (indicated by a total absence of movement, even when prodded). The concentrations cited in Table 17: Example 9 may be adjusted to meet the criteria of the specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague, 1973).

Table 17: Example 9

Test Media Concentrations (% test media)	Test Dilution Volumes (mL)	
	Diluent	Test Media
.00	500.0	0
0.1	499.5	0.5
1.0	495.0	5.0
10	450.0	50.0
50	250.0	250.0
100	0	500.0

2. The example below provides enough test medium for five replicates containing 80 mL each and extra for chemical analysis. Other ranges may be used according to needs of the analyses.

3. Rinse all exposure chambers, except the chamber containing 100% test medium, in dilution water before the start of the test.

4. Draw 0.5 mL of the test medium into a volumetric flask and dilute to 500 mL. Using a graduated cylinder, pour 80 mL into each exposure chamber labeled for 0.1% test concentration and pour the rest into a beaker for chemical measurements.

5. Continue step 4 for all concentrations. Always work from the lowest concentration to the highest in order to minimize the risk of cross-contamination.

6. Using a wide-bore pipette, randomly select and carefully place one daphnid into each exposure chamber by placing the pipette tip below the surface and gently expelling the daphnid into the chamber.

7. The test begins when half of the organisms are in the exposure chambers.

8. Concentrations are renewed every other day for the duration of the test. However, if the test begins on a Monday, then renewals may be done on Wednesday, Friday and the following Monday and Wednesday.

9. Measure and record mortality and survival at 1 hour and then when test concentrations are renewed. Count the number of live or dead young produced by each female.

10. Measure temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness of all new concentrations. Conduct these measurements on old test concentrations at least three times during the test.

11. Prepare test medium concentrations as done previously. Pour the concentrations into new exposure chambers, reserving extra for chemical analyses.

12. Count the number of live or dead adults and young, using a light table if necessary.

13. Record these results and then carefully transfer the adult daphnid into the new concentrations.

14. Using a suitable food, feed daphnids once daily during the test.

15. After feeding the daphnids, cover the exposure chamber to reduce evaporation of the test concentrations.

9.8 CALCULATIONS

The methods used to determine the EC_{50} differ depending on the results of the test. If there is no partial mortality in any replicate (i.e. all alive or all dead), then the Moving-Average Method may be used to determine the EC_{50}. If there is partial mortality within a replicate, then the Probit Method should be used to calculate the EC_{50}. Also the Lowest Observable Effect Concentration (LOEC) is recorded and the No Observable Effects Concentration (NOEC) is recorded (Peltier and Weber, 1985). Dunnett's many-one t procedure or Bonferroni's t procedure (Miller, 1966) may be used to determine comparisons between the organisms's response to the test medium concentrations as compared to the control. Other methods of estimating the response values may be used if justified and an accepted reference is cited (Biesinger, et al. 1987).

9.9 QUALITY ASSURANCE/ QUALITY CONTROL

Follow the guidelines in this SOP, which are summarized in Table 18, for adequate QA/QC.

9.10 DATA VALIDATION

Test data is invalidated for the following reasons:

- Greater than 20% control mortality.
- Standard reference toxicant results greater than two standard deviations from an accepted value (American Public Health Association, 1985).
- Greater than 20% aberrant mortality in concentrations.
- Temperature variation greater than 2°C.
- Test medium stored more than 72 hours.
- Criteria in Table 18 not met.

9.11 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, OSHA and specific health and safety procedures.

Table 18: Summary of Test Conditions for a 10-Day Chronic Toxicity Test using *Daphnia magna* or *Daphnia pulex**

1.	Test type	Static, renewal
2.	Temperature	25.0°C ± 2°C
3.	Light quality	Ambient laboratory illumination
4.	Light intensity	50-100 foot candles
5.	Photoperiod	16 hours light, 8 hours dark
6.	Test chamber size	100-mL containers
7.	Test solution volume	80 mL/replicate
8.	Renewal	Every other day
9.	Age of test organisms	Less than 24 hours old
10.	Number/container	1 per exposure chamber
11.	Feeding	Feed on day of renewal
12.	Aeration	None unless DO concentration falls below 40% saturation, then <100 bubbles per minute
13.	Dilution water	Moderately hard, reconstituted, deionized water
14.	Test media/leachate concentrations	Minimum of 5 and 1 control
15.	Test duration	10 days
16.	Effects measured	Survival, growth, and reproduction

* Based on Horning and Weber, 1975.

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*U.S. Government Printing Office: 1991 — 548-187/40581