96-HOUR ACUTE TOXICITY TESTING USING PIMEPHALES PROMELAS

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* These sections affected by Revision 1.0.

SUPERCEDES: SOP #2022, Revision 0.0; 12/14/00.

1.0 SCOPE AND APPLICATION

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

These are standard (i.e., typically applicable) operating procedures which may be varied or changed as required, dependent on site conditions, equipment limitations, or limitations imposed by the procedure. In all instances, the ultimate procedures employed must be documented and associated with the final report.

Mention of trade names or commercial products does not constitute United States Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

2.0 METHOD SUMMARY

Juvenile *Pimephales promelas (P. promelas)* are exposed to site samples, or a concentration gradient of a test media over a 96-hour period. Mortality data are used to determine the acute toxicity (as Lethal Concentration [LC_{50}]) of the test media.

3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental matrix will be sampled utilizing the procedures detailed in the Environmental Response / Scientific, Engineering, Response and Analytical Services (ERT/SERAS) Standard Operating Procedures (SOP) #2013, *Surface Water Sampling*, #2016, *Sediment Sampling*, *#2012, Soil Sampling,* SERAS SOP #2003, *Sample Storage, Preservation and Handling,* and any other procedure applicable for the media sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because test organisms will be directly 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, or at 0.1 to 4 degrees Celsius (°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.

4.0 INTERFERENCES AND POTENTIAL PROBLEMS

1. This method is less sensitive than a flow-through toxicity test and the sensitivity is dependent on the accuracy of the dilutions (Weber 1993).

2. Non-target chemicals (e.g., residual chlorine) cause adverse effects to organisms, giving false results.

3. Dissolved oxygen depletion due to biological oxygen demand, chemical oxygen demand, and metabolic wastes are also potential problems.

4. Loss of toxicant through volatilization and adsorption to exposure chambers may occur (Weber 1993).

5.0 EQUIPMENT

5.1 Apparatus

1. Approximately 150 juvenile *P. promelas*, less than 14 days old (24 hour range in age)
2. 12 exposure chambers, 1 liter (L) glass, high density polyethylene (HDPE), or polypropylene (PP), labeled
3. 6 mixing chambers, 2 L
4. Graduated cylinders, 1L, 100 milliliter (mL), and 10 mL
5. Fine mesh nylon net, Nytex screen, or similar
6. Temperature controlled chamber (incubator, water bath, etc.)
7. Dilution water, 6 L/day
8. Test media, 4 L/day
9. Dissolved oxygen (DO) meter
10. pH meter
11. Conductivity meter
12. Thermometer
13. Newly hatched *Artemia* sp. (San Francisco Bay, or similar) nauplii, for food

5.2 Test Organisms

P. promelas may be reared in-house or purchased from reputable scientific vendors. Test fish from purchased batches that have exhibited more than ten percent mortality after shipping are considered unfit. Positive identification of the species must be made prior to beginning the test.

Fish selected for acclimation need to be similar in size, not more than 1.5 times the length of each other. Juvenile *P. promelas* may be fed before and during the acclimation period, and approximately two hours prior to the test solution renewal at 48 hours. Newly hatched brine shrimp nauplii (*Artemia* sp.), that have been rinsed with fresh water, should be used as food.

Since culture and testing are usually performed in the same dilution water (U.S. EPA moderately hard, reconstituted water, see Weber 1993), acclimation is usually not necessary. When alternate dilution waters are required by the test method, test fish should be acclimated to the dilution water for at least 24 hours prior to beginning of the test. Use a small pump and drip the dilution water into the acclimation chamber so that the entire volume of water is replaced over a 24-hour period. Leave the fish in this tank for another 24 hours to complete the acclimation. During this time, adjust the temperature to 20°C. Weber (1993) and Denny (1987) provide more detail and information including culturing, care, handling, and disease prevention of fathead minnows.

5.3 Equipment for Physical/Chemical Analysis

1. Properly calibrated laboratory meters are required for the measurement of DO, temperature, pH, and conductivity. Meters must have calibration checked according to the manufacturers instructions prior to use.

2. DO meter calibration must be checked daily (or prior to use), and must be verified at least weekly (or prior to use) via the Winkler method (American Public Health Association, 1992).

3. pH meters must have calibration verified daily (or prior to use), using certified pH buffers. During instrument use, calibration must be verified every three hours, to ensure that it has not drifted.

4. Conductivity meters must have calibration verified daily (or prior to use), using certified conductivity standards.

5. Thermometers and temperature recording devices must have calibration verified at least annually against a National Institute of Standards and Technology (NIST) traceable thermometer.

6. Alkalinity and hardness should be measured in the laboratory dilution water and in the highest tested concentration of the test media, at test initiation, and upon test renewal with fresh test solution.

7. All physical/chemical measurements must be recorded on properly labeled laboratory data sheets, or in a laboratory notebook.

6.0 REAGENTS

1. Dilution Water

Dilution water shall be U.S. EPA moderately hard, reconstituted water, unless otherwise specified. See Weber (1993) for the preparation of synthetic fresh water. For tests utilizing another source of water as diluent, a second laboratory control shall be set up, using U.S. EPA moderately hard, reconstituted water.

2. Test Media

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

7.0 PROCEDURES

1. Select a range of concentrations to bracket the expected toxic range (if possible). Optimally, the test concentrations should span those causing no adverse effects, to those causing adverse effects in all exposed organisms. The example concentrations listed below may be adjusted to meet the needs of a specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague 1973). The example below provides enough test media for two replicates containing 500 mL each.

Example 1. Test Dilutions

Test concentration (% Test Media)	Volume (mL) Diluent	Test media
0	1000.00	0

6.25	937.50	62.50
12.50	875.00	125.00
25.00	750.00	250.00
50.00	500.00	500.00
100.00	0	1000.00

2. Pour the appropriate volumes of dilution water and test media into each of six clean, labeled, pre-rinsed mixing chambers (one control and five test concentrations).

3. After all the test solutions have been prepared, they should be monitored for temperature, pH, DO, and conductivity. Alkalinity and hardness measurements should be made on the control and the highest tested concentration. All results must be recorded on properly labeled data sheets or lab notebooks.

4. After monitoring, the test solutions can be dispensed to clean, labeled, pre-rinsed exposure chambers. Bring the test solutions to 20°C, prior to adding the fish, to avoid thermal stress.

5. The test fish should be pooled, and placed one or two at a time into each randomly arranged test chamber (see Lewis et al. 1994 for a discussion of randomization) or into an intermediate container, in sequential order, until each container holds ten fish. Fish are transferred using either a wide bore pipette, or a soft, fine mesh nylon screen. Fish should be placed into the test chamber, below the surface of the water, to prevent entrapment in the surface film. Addition of fish signals test initiation.

6. The test chambers should be placed into a temperature controlled area (incubator, water bath, temperature controlled room, etc.) at 20°± 1°C. Test chambers should be positioned in a random order (see Lewis et al. 1994 for a discussion of randomization).

7. Mortality should be noted one hour after test initiation, and daily thereafter. Fish are considered to be dead when they are motionless (including lack of opercular movement) and do not respond to gentle prodding at the caudal peduncle. Dead fish should be recorded on the data sheets and removed to prevent fouling of the test solution.

8. Feed juvenile fish prior to test set up, and at approximately two hours before the 48 hour test solution renewal, using a freshwater rinsed, concentrated suspension of newly hatched brine shrimp (*Artemia* sp.). Due to their small naupliar size, *Artemia* from San Francisco Bay are preferred. However, other strains of *Artemia* may also be satisfactory.

9. *Artemia* should be fed at approximately 500 to 1000 nauplii (approximately 0.1 mL of a dense solution) to each chamber. Since *Artemia* will die within a few hours in fresh water, excess food should be siphoned out, to avoid potential water quality problems which may bias test results.

10. Renewal solutions should be prepared daily, as per the original test solutions. Test solutions can be exchanged by either siphoning or carefully pouring off 80 to 90 percent of the old test solution and gently refilling the test chambers with fresh solution. An attempt should be made to siphon off as much particulate waste as possible, while leaving sufficient volume to cover the test fish.

11. Old solutions should be siphoned or gently poured off (to avoid adding aeration) into a separate container for measurement of pH and DO. The replicates of each concentration may be pooled.

12. Prior to adding the fresh test solution to the test chambers, it should be brought to test temperature (20°C), to avoid introducing thermal stress.

13. At test termination, surviving fish from each test chamber should be enumerated, and the final pH and DO should be measured.

8.0 CALCULATIONS

The common statistical endpoints of the 96 hour acute toxicity test using *P. promelas* differ depending on the results of the test. For lethality endpoints LC_{50} or Effective Concentration [EC_{50}]), if there are no partial responses in any replicate (i.e., all alive or all dead), then the Graphical Interpolation Method may be used. If there are two or more test concentrations exhibiting a partial response, and the data exhibits a significant chi square test, the Probit Method should be used. If there are one or more test concentrations exhibiting partial responses, and the chi square test is not significant, the Spearman-Karber Method or the Trimmed Spearman-Karber Method may be used. All four of these methods are detailed in Weber (1993). Computer programs for Probit Analysis and Spearman-Karber are also available from U.S. EPA's internet site (http://www.epa.gov/nerleerd/stat2.htm) Other methods may be used if justified, and if the appropriate reference is cited.

9.0 QUALITY ASSURANCE/QUALITY CONTROL

Satisfactory lab performance is demonstrated by performing at least one acceptable Standard Reference Toxicant (SRT) test per month. For a given test method, successive tests must be performed with the same SRT, at the same concentrations, in the same dilution water, using the same data analysis methods. For fish cultured in-house, SRT tests should be performed at least monthly (in accordance with ERT/SERAS SOP #2020, 7-Day Standard Reference Toxicant Test Using Larval *Pimephales promelas*), with the results recorded on a properly developed control chart. For fish purchased from an outside vendor, SRT testing should be performed on every batch received.

A control chart must developed for each test species, SRT, and set of test conditions. A series of five SRT test results is satisfactory to develop a control chart. The typical endpoint recorded for *P. promelas* is the LC_{50}. Since this is a point estimate, the control chart is based on the cumulative mean of the LC_{50} results, with the upper and lower acceptability limits being two standard deviations above or below the mean. Control chart limits are recalculated with each successive test result, after 20 SRT data points have been collected, the control chart values should be maintained using only the 20 most recent data points (Lewis et al. 1994, Lee 1980).

If the toxicity value yielded by a SRT test falls outside the established control limits, the sensitivity of the organisms and the overall credibility of the test system are suspect (Lewis et al. 1994). In this case, the test procedure should be examined for defects, and testing should be repeated with another batch of fish.

Any toxicity studies performed for environmental samples at, or near the same time as a suspect SRT test should also be considered suspect. In some cases, the observed toxicity in the environmental sample may be actual, but it could also be a result of hyper- or hyposensitive test organisms. Suspect toxicity studies should be repeated. If it is not possible to repeat a study, all results should be viewed with caution, and any citation of such results must be footnoted with an explanation.

10.0 DATA VALIDATION

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

1. Greater than 10 percent control mortality
2. Greater than 10 percent non-concentration related mortality in any concentration
3. Temperature variation greater than 1°C
4. Test media stored in excess of holding time
5. Criteria in Appendix A not met
6. Concurrent SRT test endpoint falls outside of established control limits.

11.0 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, Occupational Safety and Health Administration (OSHA), and corporate health and safety procedures.

12.0 REFERENCES

American Public Health Association. 1992. Standard Methods for the Examination of Water and Wastewater. APHA, 18th Ed.

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ERT/SERAS SOP #2013, *Surface Water Sampling*.

ERT/SERAS SOP #2016, *Sediment Sampling*.

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Lee, D.R. 1980. Reference Toxicants in Quality Control of Aquatic Bioassays. In: Aquatic Invertebrate Bioassays. ASTM STP 715. A.L. Buikema and J. Cains (eds.). American Society for Testing and Materials.

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Sprague, J.B. 1973. The ABC's of Pollutant Bioassay Using Fish in Biological Methods for the Assessment of Water Quality. ASTM STP 528. American Society for Testing and Materials, pp. 6-30.

Weber, C.I., 1993. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* 4th Edition. EPA/600/4-90/027F. Environmental Monitoring Systems Laboratory, Cincinnati, OH. 293 pp.

APPENDIX A
Summary of Conditions for The 96 Hour Acute Toxicity Test Using *Pimephales promelas*
SOP #2022
June 2002

Summary of Conditions for the 96-Hour Acute Toxicity Test Using *Pimephales promelas*

1.	Test type:	Static, daily renewal
2.	Temperature:	20° ± 1°C
3.	Light quality:	Ambient laboratory illumination, cool white fluorescent
4.	Light intensity:	50 to 100 foot candles
5.	Photoperiod:	16 hours light, 8 hours dark
6.	Test chamber size:	1L beaker (glass or plastic)
7.	Test solution volume:	500 mL/replicate
8.	Renewal:	Daily
9.	Age of test organisms:	≤14 days old, 24 hour range in age
10.	Fish/test chamber:	10 per container
11.	Replicates:	Minimum of two (if comparing 100% surface water to a control, use a minimum of four replicates)
12.	Feeding:	Feed prior to test initiation, and at approximately two hours prior to the 48 hour renewal
13.	Aeration:	None unless DO concentration falls below 40% saturation; then < 100 bubbles per minute
14.	Dilution water:	U.S. EPA moderately hard reconstituted water, unless otherwise specified
15.	Test media concentrations:	Minimum of five and one control
16.	Test duration:	96 hours
17.	Test acceptability:	≥90% control survival