STANDARD OPERATING PROCEDURES

**7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas***

CONTENTS

These sections affected by Revision 1.0.

SUPERCEDES: SOP #2026, Revision 0.0; 12/14/00. EPA Contract No. EP-W-09-031.

7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas*

1.0   SCOPE AND APPLICATION

The procedure for conducting a 7-day static renewal toxicity test using larval *Pimephales promelas* (Fathead Minnows) is described. This test is applicable to effluents, leachates, and sediments, which require a chronic toxicity estimate. This method uses growth and mortality as endpoints.

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

Larval *Pimephales promelas (P. promelas)* are exposed to different concentrations of a test media over a seven day period. Survival and growth results are used to determine the No Observable Adverse Effect Concentration (NOAEC), the Lowest Observable Adverse Effect Concentration (LOAEC), and the Inhibition Concentration (IC_{25}) of the test media.

3.0   SAMPLING, PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental matrix will be sampled and handled utilizing the methods detailed in Environmental Response Team/Scientific, Engineering, Response, and Analytical Services (ERT/SERAS) Standard Operating Procedures (SOP) #2012, *Soil Sampling*, #2013, *Surface Water Sampling*, #2016, *Sediment 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 the sample material, or exposed to an elutriate of the test 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 ($^{\circ}$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.   Non-target compounds (e.g., residual chlorine) may cause adverse effects to test organisms, giving false results.

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

3.   Loss of toxicants through volatilization or adsorption to exposure chambers may occur (Weber 1993).

5.0   EQUIPMENT/APPARATUS

STANDARD OPERATING PROCEDURES

SOP: 2026
PAGE: 3 of 10
REV: 1.0
DATE: 06/24/02

**7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas***

5.1   Apparatus

1.   Approximately 250 *P. promelas*, less than or equal to ($\leq$) 24 hours old
2.   24 exposure chambers, 500 milliliters (mL) to 1 Liter (L) - glass, polypropylene (PP), or high density polyethylene (HDPE)
3.   6 mixing chambers 1 L or larger
4.   Nitex screen, approximately 500 microns (μ) mesh (or similar)
5.   Temperature controlled chamber (incubator, water bath, etc.)
6.   Dilution water - approximately 6 L/day
7.   Graduated cylinders - 500 mL, 100 mL and 10 mL
8.   Dissolved Oxygen (DO) meter
9.   pH meter
10. Conductivity meter
11. Thermometer
12. Light table (optional) - to aid in counting the organisms
13. Newly hatched *Artemia* sp. (San Francisco Bay, or similar) nauplii, for food
14. Waste containers
15. Oven, capable of maintaining 60°C for 24 hours or 100°C for 6 hours.
16. Weighing dishes

5.2   Test Organisms

Test organisms 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 for testing, and must not be used.

P. promelas larvae destined for use in testing should be less than 24 hours old. However, when larval fish are purchased from an outside vendor and must be shipped to the laboratory, the fish must be less than 48 hours old, with all fish hatching within the same 24 hour period.

To ensure larvae less than 24 hours old, use eggs that were laid approximately 3 to 4 days prior to test initiation. Place the substrate containing the eggs into a chamber containing U.S. EPA moderately hard, reconstituted water. This allows the test fish to become acclimated to the dilution water, reducing stress. Aerate the eggs gently to reduce the potential for fungal growth, and use populations of fish that have less than 5% mortality (American Public Health Association, 1992). Weber (1993) and Denny (1987) provide more detail and information including culturing, care, handling, and disease prevention of *P. promelas*.

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

STANDARD OPERATING PROCEDURES

SOP: 2026
PAGE: 4 of 10
REV: 1.0
DATE: 06/24/02

**7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas***

(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's 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 (e.g., surface water), 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 or soil, 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 four replicates containing 250 mL each. Other concentration ranges may be used at the investigators discretion.

Example 1 Test Dilutions

7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas*

Test concentration (% Test Media)	Volume (mL) Diluent	Volume (mL) Test media
0.00	1000.00	0.00
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	0.00	10000.00

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 in laboratory notebooks.

4. After monitoring, the test solutions can be dispensed to clean, labeled, pre-rinsed exposure chambers, and the test fish may be added. The test solutions must be brought to test temperature (25 °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. A minimum of four replicate test chambers should be used for each exposure treatment. 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. The addition of fish to the test chambers signals test initiation.

6. The test chambers should be placed into a temperature controlled area (incubator, water bath, temperature controlled room, etc.) at 25 ° ± 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 larval fish two or three times daily at four to six hour intervals, using a freshwater-rinsed, concentrated suspension of newly hatched brine shrimp (*Artemia* sp.). Do not feed test fish for the last twelve hours prior to test termination.

9. Due to their small naupliar size, *Artemia* from San Francisco Bay are preferred. However, other strains may also be used. *Artemia* should be fed at approximately 500 to 1000 nauplii (about 0.1 mL of a dense solution) to each chamber. Excess food should be siphoned out as soon as possible, at a minimum once per day, to avoid water quality problems, which may interfere with test results.

7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas*

10. New exposure solutions must be prepared daily. Test solutions can be exchanged by either siphoning or carefully pouring off 80 to 90% of the old test solution and gently refilling the test chambers with fresh solution. Care should be taken to remove as much particulate waste as possible, while leaving sufficient volume to cover the test fish.

11. Old solutions should be 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. Replace the old solution with new solution by gently pouring the new solution down the sides of the test chamber, taking care not to agitate the test fish. Prior to adding the fresh test solution to the test chambers, it should be brought to test temperature (25°C), to avoid introducing thermal stress.

13. At test termination, surviving fish from each test chamber should be enumerated, poured into a fine mesh net and rinsed with deionized water. The rinsed fish should be counted into tared weighing dishes and dried in a drying oven (at 60°C for 24 hours, or at 100°C for at least six hours). The dried fish can then be cooled in a dessicator, and weighed to the nearest 0.1 milligrams (mg).

8.0   CALCULATIONS

The common statistical endpoints of the short-term chronic toxicity test using *P. promelas* differ depending on the results of the test. For lethality endpoints (LC_{50}, 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 partial responses, 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).

The Lowest Observable Adverse Effect Concentration (LOAEC), and the No Observable Adverse Effect Concentration (NOAEC) are calculated for both survival and growth. The LOAEC/NOAEC for survival is calculated first. Prior to determining the LOAEC/NOAEC for survival, the data set is checked for normality using the Shapiro-Wilk's Test. If the data set is normally distributed, it is then checked for homogeneity of variance, using Bartlett's Test. If the data set meets the assumptions of normality and homogeneity of variance, it is tested for LOAEC/NOEAC using Dunnett's Test for data sets with equal number of replicates, or the Bonferroni T-test for data sets with unequal number of replicates. If the data set does not meet the assumptions of normality and homogeneity of variance, it is tested for LOAEC/NOAEC using the Steel's Many-One Rank Test for data sets with equal number of replicates, or the Wilcoxon Rank Sum Test with Bonferroni Adjustment for data sets with unequal number of replicates. All of these methods are detailed in Lewis et al. (1994). Several commercially available computer programs will also perform these calculations. Test concentrations exhibiting significant difference from the control (the LOAEC and higher concentrations) are not used for calculation of growth effects.

Prior to determining the LOAEC/NOAEC for growth, the data set (excluding data with significantly affected survival) is checked for normality using the Shapiro-Wilk's Test. If the data set is normally distributed, it is then checked for homogeneity of variance, using Bartlett's Test. If the data set meets the assumptions of

7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas*

---

normality and homogeneity of variance, it is tested for LOAEC/NOEAC using Dunnett's Test for data sets with equal number of replicates, or the Bonferroni T-test for data sets with unequal number of replicates. If the data set does not meet the assumptions of normality and homogeneity of variance, it is tested for LOAEC/NOAEC using the Steel's Many-One Rank Test for data sets with equal number of replicates, or the Wilcoxon Rank Sum Test with Bonferroni Adjustment for data sets with unequal number of replicates. All of these methods are detailed in Lewis et al. (1994). Several commercially available computer programs will also perform these calculations.

The Inhibition Concentration (IC) at which there is a reduction in growth, usually calculated for a 25% reduction (IC_{25}), is calculated using the U.S. EPA's IC_p program. The program is available from U.S. EPA Duluth, or it can be downloaded from U.S. EPA's site on the internet (http://www.epa.gov/nerleerd/stat2.htm).

9.0   QUALITY ASSURANCE/QUALITY CONTROL

Satisfactory laboratory performance is demonstrated by performing at least one acceptable Standard Reference Toxicant (SRT) test per month (see ERT/SERAS SOP 2020, *7-Day Standard Reference Toxicant Test Using Larval Pimephales promelas*). 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, 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 be 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 IC_{25} for growth. Since this is a point estimate endpoint, the control chart is based on the cumulative mean of the IC_{25} 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).

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 deviations, 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 are also considered to be 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 criteria below are a basis for rejecting test results:

1.   Greater than 20 percent control mortality.
2.   Greater than 20 percent non-concentration related mortality in any test concentration.

STANDARD OPERATING PROCEDURES

SOP: 2026
PAGE: 8 of 10
REV: 1.0
DATE: 06/24/02

7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas*

3. Control fish weights averaging less than 0.25 mg.
4. Temperature variation greater than $25 \pm 2°C$.
5. Concurrent SRT test endpoint falls outside of established control limits.
6. Criteria in Appendix A not met.

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 edition.

Denny, J.S. 1987. *Guidelines for the Culturing of Fathead Minnows for use in Toxicity Tests*. EPA/600/3-87/001. Environmental Research Laboratory, Duluth, MN. 49 pp.

SERAS SOP #2003, *Sample Storage, Preservation and Handling*.

ERT/SERAS SOP #2012, *Soil Sampling*.

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

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

ERT/SERAS SOP #2020, *7-Day Standard Reference Toxicant Test Using Larval Pimephales promelas*.

Lewis, P.A., D.J. Klemm, J.M. Lazorchak, T.J. Norberg-King, W.H. Peltier, M.A. Heber. 1994. *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*. Third Edition. EPA/600/4-91/002. Environmental Monitoring Systems Laboratory, Cincinnati, OH. 341 pp.

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.

STANDARD OPERATING PROCEDURES

SOP: 2026
PAGE: 9 of 10
REV: 1.0
DATE: 06/24/02

**7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas***

APPENDIX A
Summary of Conditions for Larval *Pimephales promelas* Survival and Growth Test
SOP #2026
June 2002

**7-DAY STATIC RENEWAL TOXICITY TEST
USING LARVAL *Pimephales promelas***

Summary of Conditions for Larval *Pimephales promelas* Survival and Growth Test

1	Test type	Static; daily renewal
2	Temperature:	$25° \pm 1°C$
3	Illumination:	Cool white fluorescent lights, approximately 50 to 100 foot-candles at the test surface
4	Photoperiod:	16 hours light, 8 hours dark
5	Test chamber size:	500 mL containers (or similar)
6	Test solution volume:	250 mL per exposure chamber
7	Renewal:	Daily
8	Age of test organisms:	Newly hatched larvae $\leq$ 24 hours old; if shipped, not more than 48 hours old, all hatched within 24 hours
9	Replication:	Ten organisms per replicate Four replicates per concentration
10	Feeding:	Feed approximately 0.1 gram (g) newly hatched *Artemia* nauplii two or three times per day. No feeding during the final 12 hours of the test.
11	Aeration:	None, unless the DO falls below 4.0 milligram per liter (mg/L). Rate should not exceed 100 bubbles per minute.
12	Dilution water:	U.S. EPA moderately hard reconstituted water, unless otherwise specified
13	Test media concentrations:	Minimum of five and one control
14	Test duration:	Seven days
15	Effects measured:	Survival and growth (as dry weight)
16	Test acceptability:	$\geq$80% control survival Surviving controls average $\geq$0.25 mg per fish