MARINE ACUTE

TOXICITY TEST PROCEDURE AND PROTOCOL

I. GENERAL REQUIREMENTS

The permittee shall conduct acceptable acute toxicity tests in accordance with the appropriate test protocols described below:

- **2007.0 - Mysid Shrimp (Americamysis bahia) definitive 48 hour test.**
- **2006.0 - Inland Silverside (Menidia beryllina) definitive 48 hour test.**

Acute toxicity data shall be reported as outlined in Section VIII.

II. METHODS

The permittee shall use the most recent 40 CFR Part 136 methods. Whole Effluent Toxicity (WET) Test Methods and guidance may be found at:

http://water.epa.gov/scitech/methods/cwa/wet/index.cfm#methods

The permittee shall also meet the sampling, analysis and reporting requirements included in this protocol. This protocol defines more specific requirements while still being consistent with the Part 136 methods. If, due to modifications of Part 136, there are conflicting requirements between the Part 136 method and this protocol, the permittee shall comply with the requirements of the Part 136 method.

III. SAMPLE COLLECTION

A discharge and receiving water sample shall be collected. The receiving water control sample must be collected immediately upstream of the permitted discharge's zone of influence. The acceptable holding times until initial use of a sample are 24 and 36 hours for on-site and off-site testing, respectively. A written waiver is required from the regulating authority for any holding time extension. Sampling guidance dictates that, where appropriate, aliquots for the analysis required in this protocol shall be split from the samples, containerized and immediately preserved, or analyzed as per 40 CFR Part 136. EPA approved test methods require that samples collected for metals analyses be preserved immediately after collection. Testing for the presence of total residual chlorine[1] (TRC) must be analyzed immediately or as soon as possible, for all effluent samples, prior to WET testing. TRC analysis may be performed on-site or by the toxicity testing laboratory and the samples must be dechlorinated, as necessary, using sodium thiosulfate

[1] For this protocol, total residual chlorine is synonymous with total residual oxidants.

prior to sample use for toxicity testing. If performed on site the results should be included on the chain of custody (COC) presented to WET laboratory.

Standard Methods for the Examination of Water and Wastewater describes dechlorination of samples (APHA, 1992). Dechlorination can be achieved using a ratio of 6.7 mg/L anhydrous sodium thiosulfate to reduce 1 mg/L chlorine. If dechlorination is necessary, a thiosulfate control consisting of the maximum concentration of thiosulfate used to dechlorinate the sample in the toxicity test control water must also be run in the WET test.

All samples submitted for chemical and physical analyses will be analyzed according to Section VI of this protocol. Grab samples must be used for pH, temperature, and total residual chlorine (as per 40 CFR Part 122.21).

All samples held for use beyond the day of sampling shall be refrigerated and maintained at a temperature range of 0-6^{o} C.

IV. DILUTION WATER

Samples of receiving water must be collected from a reasonably accessible location in the receiving water body immediately upstream of the permitted discharge's zone of influence. Avoid collection near areas of obvious road or agricultural runoff, storm sewers or other point source discharges and areas where stagnant conditions exist. EPA strongly urges that screening for toxicity be performed prior to the set up of a full, definitive toxicity test any time there is a question about the test dilution water's ability to achieve test acceptability criteria (TAC) as indicated in Section V of this protocol. The test dilution water control response will be used in the statistical analysis of the toxicity test data. All other control(s) required to be run in the test will be reported as specified in the Discharge Monitoring Report (DMR) Instructions, Attachment F, page 2,Test Results & Permit Limits.

The test dilution water must be used to determine whether the test met the applicable TAC. When receiving water is used for test dilution, an additional control made up of standard laboratory water (0% effluent) is required. This control will be used to verify the health of the test organisms and evaluate to what extent, if any, the receiving water itself is responsible for any toxic response observed.

If dechlorination of a sample by the toxicity testing laboratory is necessary a "sodium thiosulfate" control, representing the concentration of sodium thiosulfate used to adequately dechlorinate the sample prior to toxicity testing, must be included in the test.

If the use of alternate dilution water (ADW) is authorized, in addition to the ADW test control, the testing laboratory must, for the purpose of monitoring the receiving water, also run a receiving water control.

If the receiving water is found to be, or suspected to be toxic or unreliable, ADW of known quality with hardness similar to that of the receiving water may be substituted. Substitution is

species specific meaning that the decision to use ADW is made for each species and is based on the toxic response of that particular species. Substitution to an ADW is authorized in two cases. The first case is when repeating a test due to toxicity in the site dilution water requires an **immediate decision** for ADW use by the permittee and toxicity testing laboratory. The second is when two of the most recent documented incidents of unacceptable site dilution water toxicity require ADW use in future WET testing.

For the second case, written notification from the permittee requesting ADW use **and** written authorization from the permit issuing agency(s) is required **prior to** switching to a long-term use of ADW for the duration of the permit.

Written requests for use of ADW must be mailed with supporting documentation to the following addresses:

Director
Office of Ecosystem Protection (CAA)
U.S. Environmental Protection Agency, Region 1
Five Post Office Square, Suite 100
Mail Code OEP06-5
Boston, MA 02109-3912

and

Manager
Water Technical Unit (SEW)
U.S. Environmental Protection Agency
Five Post Office Square, Suite 100
Mail Code OES04-4
Boston, MA 02109-3912

Note: USEPA Region 1 retains the right to modify any part of the alternate dilution water policy stated in this protocol at any time. Any changes to this policy will be documented in the annual DMR posting.

See the most current annual DMR instructions which can be found on the EPA Region 1 website at http://www.epa.gov/region1/enforcementandassistance/dmr.html for further important details on alternate dilution water substitution requests.

V. TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

EPA Region 1 requires tests be performed using four replicates of each control and effluent concentration because the non-parametric statistical tests cannot be used with data from fewer replicates. The following tables summarize the accepted Americamysis and Menidia toxicity test conditions and test acceptability criteria:

EPA NEW ENGLAND EFFLUENT TOXICITY TEST CONDITIONS FOR THE MYSID, AMERICAMYSIS BAHIA 48 HOUR TEST[1]

1. Test type	48hr Static, non-renewal
2. Salinity	25ppt ± 10 percent for all dilutions by adding dry ocean salts
3. Temperature (^{o}C)	20^{o}C ± 1^{o}C or 25^{o}C ± 1^{o}C, temperature must not deviate by more than 3^{o}C during test
4. Light quality	Ambient laboratory illumination
5. Photoperiod	16 hour light, 8 hour dark
6. Test chamber size	250 ml (minimum)
7. Test solution volume	200 ml/replicate (minimum)
8. Age of test organisms	1-5 days, < 24 hours age range
9. No. Mysids per test chamber	10
10. No. of replicate test chambers per treatment	4
11. Total no. Mysids per test concentration	40
12. Feeding regime	Light feeding using concentrated Artemia naupli while holding prior to initiating the test
13. Aeration [2]	None
14. Dilution water	5-30 ppt, +/- 10%; Natural seawater, or deionized water mixed with artificial sea salts
15. Dilution factor	≥ 0.5
16. Number of dilutions [3]	5 plus a control. An additional dilution at the permitted effluent concentration (%

	effluent) is required if it is not included in the dilution series.
17. Effect measured	Mortality - no movement of body appendages on gentle prodding
18. Test acceptability	90% or greater survival of test organisms in control solution
19. Sampling requirements	For on-site tests, samples are used within 24 hours of the time that they are removed from the sampling device. For off-site tests, samples must be first used within 36 hours of collection.
20. Sample volume required	Minimum 1 liter for effluents and 2 liters for receiving waters

Footnotes:

[1] Adapted from EPA 821-R-02-012.

[2] If dissolved oxygen falls below 4.0 mg/L, aerate at rate of less than 100 bubbles/min. Routine D.O. checks are recommended.

[3] When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

EPA NEW ENGLAND TOXICITY TEST CONDITIONS FOR THE INLAND SILVERSIDE, MENIDIA BERYLLINA 48 HOUR TEST[1]

1. Test Type	48 hr Static, non-renewal
2. Salinity	25 ppt ± 10 % by adding dry ocean salts
3. Temperature	20°C ± 1°C or 25°C ± 1°C, temperature must not deviate by more than 3°C during test
4. Light Quality	Ambient laboratory illumination
5. Photoperiod	16 hr light, 8 hr dark
6. Size of test vessel	250 mL (minimum)
7. Volume of test solution	200 mL/replicate (minimum)
8. Age of fish	9-14 days; 24 hr age range
9. No. fish per chamber	10 (not to exceed loading limits)
10. No. of replicate test vessels per treatment	4
11. Total no. organisms per concentration	40
12. Feeding regime	Light feeding using concentrated Artemia nauplii while holding prior to initiating the test
13. Aeration[2]	None
14. Dilution water	5-32 ppt, +/- 10% ; Natural seawater, or deionized water mixed with artificial sea salts.
15. Dilution factor	≥ 0.5
16. Number of dilutions[3]	5 plus a control. An additional dilution at the permitted concentration (% effluent) is required if it is not included in the dilution series.
17. Effect measured	Mortality-no movement on gentle prodding.

18. Test acceptability	90% or greater survival of test organisms in control solution.
19. Sampling requirements	For on-site tests, samples must be used within 24 hours of the time they are removed from the sampling device. Off-site test samples must be used within 36 hours of collection.
20. Sample volume required	Minimum 1 liter for effluents and 2 liters for receiving waters.

Footnotes:

[1] Adapted from EPA 821-R-02-012.

[2] If dissolved oxygen falls below 4.0 mg/L, aerate at rate of less than 100 bubbles/min. Routine D.O. checks recommended.

[3] When receiving water is used for dilution, an additional control made up of standard laboratory dilution water (0% effluent) is required.

V.1. Test Acceptability Criteria

If a test does not meet TAC the test must be repeated with fresh samples within 30 days of the initial test completion date.

V.2. Use of Reference Toxicity Testing

Reference toxicity test results and applicable control charts must be included in the toxicity testing report.

In general, if reference toxicity test results fall outside the control limits established by the laboratory for a specific test endpoint, a reason or reasons for this excursion must be evaluated, correction made and reference toxicity tests rerun as necessary as prescribed below.

If a test endpoint value exceeds the control limits at a frequency of more than one out of twenty then causes for the reference toxicity test failure must be examined and if problems are identified corrective action taken. The reference toxicity test must be repeated during the same month in which the exceedance occurred.

If two consecutive reference toxicity tests fall outside control limits, the possible cause(s) for the exceedance must be examined, corrective actions taken and a repeat of the reference toxicity test must take place immediately. Actions taken to resolve the problem must be reported.

V.2.a. Use of Concurrent Reference Toxicity Testing

In the case where concurrent reference toxicity testing is required due to a low frequency of testing with a particular method, if the reference toxicity test results fall slightly outside of laboratory established control limits, but the primary test met the TAC, the results of the primary test will be considered acceptable. However, if the results of the concurrent test fall well outside the established **upper** control limits i.e. ≥3 standard deviations for IC25s and LC50 values and ≥ two concentration intervals for NOECs or NOAECs, and even though the primary test meets TAC, the primary test will be considered unacceptable and must be repeated.

VI. CHEMICAL ANALYSIS

At the beginning of the static acute test, pH, salinity, and temperature must be measured at the beginning and end of each 24 hour period in each dilution and in the controls. The following chemical analyses shall be performed for each sampling event.

Parameter	Effluent	Diluent	Minimum Level for effluent[*1] (mg/L)
pH	x	x	---
Salinity	x	x	ppt(o/oo)
Total Residual Chlorine [*2]	x	x	0.02
Total Solids and Suspended Solids	x	x	---
Ammonia	x	x	0.1
Total Organic Carbon	x	x	0.5
Total Metals			
Cd	x	x	0.0005
Pb	x	x	0.0005
Cu	x	x	0.003
Zn	x	x	0.005
Ni	x	x	0.005

Superscript:

[*1] These are the minimum levels for effluent (fresh water) samples. Tests on diluents (marine waters) shall be conducted using the Part 136 methods that yield the lowest MLs.

[*2] Either of the following methods from the 18th Edition of the APHA Standard Methods for the Examination of Water and Wastewater must be used for these analyses:

-Method 4500-Cl E Low Level Amperometric Titration (the preferred method);
-Method 4500-CL G DPD Photometric Method.

VII. TOXICITY TEST DATA ANALYSIS

LC50 Median Lethal Concentration

An estimate of the concentration of effluent or toxicant that is lethal to 50% of the test organisms during the time prescribed by the test method.

Methods of Estimation:

- Probit Method
- Spearman-Karber
- Trimmed Spearman-Karber
- Graphical

See flow chart in Figure 6 on page 73 of EPA 821-R-02-012 for appropriate method to use on a given data set.

No Observed Acute Effect Level (NOAEL)

See flow chart in Figure 13 on page 87 of EPA 821-R-02-012.

VIII. TOXICITY TEST REPORTING

A report of results must include the following:

- Toxicity Test summary sheet(s) (Attachment F to the DMR Instructions) which includes:
    - Facility name
    - NPDES permit number
    - Outfall number
    - Sample type
    - Sampling method
    - Effluent TRC concentration
    - Dilution water used
    - Receiving water name and sampling location
    - Test type and species
    - Test start date
    - Effluent concentrations tested (%) and permit limit concentration
    - Applicable reference toxicity test date and whether acceptable or not
    - Age, age range and source of test organisms used for testing
    - Results of TAC review for all applicable controls
    - Permit limit and toxicity test results
    - Summary of any test sensitivity and concentration response evaluation that was conducted

Please note: The NPDES Permit Program Instructions for the Discharge Monitoring Report Forms (DMRs) are available on EPA's website at http://www.epa.gov/NE/enforcementandassistance/dmr.html

In addition to the summary sheets the report must include:

- A brief description of sample collection procedures;
- Chain of custody documentation including names of individuals collecting samples, times and dates of sample collection, sample locations, requested analysis and lab receipt with time and date received, lab receipt personnel and condition of samples upon receipt at the lab(s);
- Reference toxicity test control charts;
- All sample chemical/physical data generated, including minimum levels (MLs) and analytical methods used;
- All toxicity test raw data including daily ambient test conditions, toxicity test chemistry, sample dechlorination details as necessary, bench sheets and statistical analysis;
- A discussion of any deviations from test conditions; and
- Any further discussion of reported test results, statistical analysis and concentration-response relationship and test sensitivity review per species per endpoint.