

ATTACHMENT A

USEPA REGION 1 FRESHWATER 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:

- **Daphnid (Ceriodaphnia dubia) definitive 48 hour test.**
- **Fathead Minnow (Pimephales promelas) definitive 48 hour test.**

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

II. METHODS

The permittee shall use 40 CFR Part 136 methods. Methods and guidance may be found at:

http://water.epa.gov/scitech/methods/cwa/wet/disk2_index.cfm

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 sample shall be collected. Aliquots shall be split from the sample, containerized and preserved (as per 40 CFR Part 136) for chemical and physical analyses required. The remaining sample shall be measured for total residual chlorine and dechlorinated (if detected) in the laboratory using sodium thiosulfate for subsequent toxicity testing. (Note that EPA approved test methods require that samples collected for metals analyses be preserved immediately after collection.) Grab samples must be used for pH, temperature, and total residual chlorine (as per 40 CFR Part 122.21).

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 1.0 mg/L chlorine. If dechlorination is necessary, a thiosulfate control (maximum amount of thiosulfate in lab control or receiving water) must also be run in the WET test.

All samples held overnight shall be refrigerated at 1- 6°C.

IV. DILUTION WATER

A grab sample of dilution water used for acute toxicity testing shall be collected from the receiving water at a point immediately upstream of the permitted discharge's zone of influence at a reasonably accessible location. Avoid collection near areas of obvious road or agricultural runoff, storm sewers or other point source discharges and areas where stagnant conditions exist. In the case where an alternate dilution water has been agreed upon an additional receiving water control (0% effluent) must also be tested.

If the receiving water diluent is found to be, or suspected to be toxic or unreliable, an alternate standard dilution water of known quality with a hardness, pH, conductivity, alkalinity, organic carbon, and total suspended solids similar to that of the receiving water may be substituted **AFTER RECEIVING WRITTEN APPROVAL FROM THE PERMIT ISSUING AGENCY(S)**. Written requests for use of an alternate dilution water should be mailed with supporting documentation to the following address:

Director
Office of Ecosystem Protection (CAA)
U.S. Environmental Protection Agency-New England
5 Post Office Sq., Suite 100 (OEP06-5)
Boston, MA 02109-3912

and

Manager
Water Technical Unit (SEW)
U.S. Environmental Protection Agency
5 Post Office Sq., Suite 100 (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/enforcement/water/dmr.html> for further important details on alternate dilution water substitution requests.

It may prove beneficial to have the proposed dilution water source screened for suitability prior to toxicity testing. EPA strongly urges that screening be done prior to set up of a full definitive toxicity test any time there is question about the dilution water's ability to support acceptable performance as outlined in the 'test acceptability' section of the protocol.

V. TEST CONDITIONS

The following tables summarize the accepted daphnid and fathead minnow toxicity test conditions and test acceptability criteria:

EPA NEW ENGLAND EFFLUENT TOXICITY TEST CONDITIONS FOR THE DAPHNID, CERIODAPHNIA DUBIA 48 HOUR ACUTE TESTS¹

1.	Test type	Static, non-renewal
2.	Temperature (°C)	20 ± 1°C or 25 ± 1°C
3.	Light quality	Ambient laboratory illumination
4.	Photoperiod	16 hour light, 8 hour dark
5.	Test chamber size	Minimum 30 ml
6.	Test solution volume	Minimum 15 ml
7.	Age of test organisms	1-24 hours (neonates)
8.	No. of daphnids per test chamber	5
9.	No. of replicate test chambers per treatment	4
10.	Total no. daphnids per test concentration	20
11.	Feeding regime	As per manual, lightly feed YCT and <u>Selenastrum</u> to newly released organisms while holding prior to initiating test
12.	Aeration	None
13.	Dilution water ²	Receiving water, other surface water, synthetic water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q ^R or equivalent deionized water and reagent grade chemicals according to EPA acute toxicity test manual) or deionized water combined with mineral water to appropriate hardness.
14.	Dilution series	≥ 0.5, must bracket the permitted RWC
15.	Number of dilutions	5 plus receiving water and laboratory water control and thiosulfate control, as necessary. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution

series.

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| 16. Effect measured | Mortality-no movement of body or appendages on gentle prodding |
| 17. Test acceptability | 90% or greater survival of test organisms in dilution water control solution |
| 18. Sampling requirements | For on-site tests, samples must be used within 24 hours of the time that they are removed from the sampling device. For off-site tests, samples must first be used within 36 hours of collection. |
| 19. Sample volume required | Minimum 1 liter |

Footnotes:

1. Adapted from EPA-821-R-02-012.
2. Standard prepared dilution water must have hardness requirements to generally reflect the characteristics of the receiving water.

**EPA NEW ENGLAND TEST CONDITIONS FOR THE FATHEAD MINNOW
(PIMEPHALES PROMELAS) 48 HOUR ACUTE TEST¹**

1. Test Type	Static, non-renewal
2. Temperature (°C)	20 ± 1 ° C or 25 ± 1°C
3. Light quality	Ambient laboratory illumination
4. Photoperiod	16 hr light, 8 hr dark
5. Size of test vessels	250 mL minimum
6. Volume of test solution	Minimum 200 mL/replicate
7. Age of fish	1-14 days old and age within 24 hrs of each other
8. No. of fish per chamber	10
9. No. of replicate test vessels per treatment	4
10. Total no. organisms per concentration	40
11. Feeding regime	As per manual, lightly feed test age larvae using concentrated brine shrimp nauplii while holding prior to initiating test
12. Aeration	None, unless dissolved oxygen (D.O.) concentration falls below 4.0 mg/L, at which time gentle single bubble aeration should be started at a rate of less than 100 bubbles/min. (Routine D.O. check is recommended.)
13. dilution water ²	Receiving water, other surface water, synthetic water adjusted to the hardness and alkalinity of the receiving water (prepared using either Millipore Milli-Q ^R or equivalent deionized and reagent grade chemicals according to EPA acute toxicity test manual) or deionized water combined with mineral water to appropriate hardness.
14. Dilution series	≥ 0.5, must bracket the permitted RWC

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| 15. Number of dilutions | 5 plus receiving water and laboratory water control and thiosulfate control, as necessary. An additional dilution at the permitted effluent concentration (% effluent) is required if it is not included in the dilution series. |
| 16. Effect measured | Mortality-no movement on gentle prodding |
| 17. Test acceptability | 90% or greater survival of test organisms in dilution water control solution |
| 18. Sampling requirements | For on-site tests, samples must be used within 24 hours of the time that they are removed from the sampling device. For off-site tests, samples are used within 36 hours of collection. |
| 19. Sample volume required | Minimum 2 liters |

Footnotes:

1. Adapted from EPA-821-R-02-012
2. Standard dilution water must have hardness requirements to generally reflect characteristics of the receiving water.

VI. CHEMICAL ANALYSIS

At the beginning of a static acute toxicity test, pH, conductivity, total residual chlorine, oxygen, hardness, alkalinity and temperature must be measured in the highest effluent concentration and the dilution water. Dissolved oxygen, pH and temperature are also measured at 24 and 48 hour intervals in all dilutions. The following chemical analyses shall be performed on the 100 percent effluent sample and the upstream water sample for each sampling event.

<u>Parameter</u>	Effluent	Receiving Water	ML (mg/l)
Hardness ¹	x	x	0.5
Total Residual Chlorine (TRC) ^{2, 3}	x		0.02
Alkalinity	x	x	2.0
pH	x	x	--
Specific Conductance	x	x	--
Total Solids	x		--
Total Dissolved Solids	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
Al	x	x	0.02
Other as permit requires			

Notes:

- Hardness may be determined by:
 - APHA Standard Methods for the Examination of Water and Wastewater , 21st Edition
 - Method 2340B (hardness by calculation)
 - Method 2340C (titration)
- Total Residual Chlorine may be performed using any of the following methods provided the required minimum limit (ML) is met.
 - APHA Standard Methods for the Examination of Water and Wastewater , 21st Edition
 - Method 4500-CL E Low Level Amperometric Titration
 - Method 4500-CL G DPD Colorimetric Method
- Required to be performed on the sample used for WET testing prior to its use for toxicity testing.

VII. TOXICITY TEST DATA ANALYSIS

LC50 Median Lethal Concentration (Determined at 48 Hours)

Methods of Estimation:

- Probit Method
- Spearman-Kärber
- Trimmed Spearman-Kärber
- Graphical

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

No Observed Acute Effect Level (NOAEL)

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

VIII. TOXICITY TEST REPORTING

A report of the results will include the following:

- Description of sample collection procedures, site description
- Names of individuals collecting and transporting samples, times and dates of sample collection and analysis on chain-of-custody
- General description of tests: age of test organisms, origin, dates and results of standard toxicant tests; light and temperature regime; other information on test conditions if different than procedures recommended. Reference toxicant test data should be included.
- All chemical/physical data generated. (Include minimum detection levels and minimum quantification levels.)
- Raw data and bench sheets.
- Provide a description of dechlorination procedures (as applicable).
- Any other observations or test conditions affecting test outcome.