FRESHWATER CHRONIC
TOXICITY TEST PROCEDURE AND PROTOCOL
USEPA Region 1

I. GENERAL REQUIREMENTS

The permittee shall be responsible for the conduct of acceptable chronic (and modified acute) toxicity tests using three fresh samples collected during each test period. The following tests shall be performed as prescribed in Part 1 of the NPDES discharge permit in accordance with the appropriate test protocols described below. (Note: the permittee and testing laboratory should review the applicable permit to determine whether testing of one or both species is required).

- **Daphnid (Ceriodaphnia dubia) Survival and Reproduction Test.**

- **Fathead Minnow (Pimephales promelas) Larval Growth and Survival Test.**

Chronic and modified acute toxicity data shall be reported as outlined in Section VIII. The chronic fathead minnow and daphnid test data can be used to calculate an LC50 at the end of 48 hours of exposure when both acute (LC50) and chronic (C-NOEC) test endpoints are specified in the permit.

II. METHODS

Methods to follow are those recommended by EPA in: Short Term Methods For Estimating The Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms, Fourth Edition. October 2002. United States Environmental Protection Agency. Office of Water, Washington, D.C., EPA 821-R-02-013. The methods are available on-line at http://www.epa.gov/waterscience/WET/ . Exceptions and clarification are stated herein.

III. SAMPLE COLLECTION AND USE

A total of three fresh samples of effluent and receiving water are required for initiation and subsequent renewals of a freshwater, chronic, toxicity test. The receiving water control sample must be collected immediately upstream of the permitted discharge's zone of influence. Fresh samples are recommended for use on test days 1, 3, and 5. However, provided a total of three samples are used for testing over the test period, an alternate sampling schedule is acceptable. 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 hold time extension. All test samples collected may be used for 24, 48 and 72 hour renewals after initial use. All samples held for use beyond the day of sampling shall be refrigerated and maintained at a temperature range of 0-6^{o} C.

All samples submitted for chemical and physical analyses will be analyzed according to Section VI of this protocol.

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 (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 prior to sample use for toxicity testing.

If any of the renewal samples are of sufficient potency to cause lethality to 50 percent or more of the test organisms in any of the test treatments for either species or, if the test fails to meet its permit limits, then chemical analysis for total metals (originally required for the initial sample only in Section VI) will be required on the renewal sample(s) as well.

IV. DILUTION WATER

Samples of receiving water must be collected from a location in the receiving water body 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. 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 an 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 diluent is found to be, or suspected to be toxic or unreliable an 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 is the case where repeating a test due to toxicity in the site dilution water requires an **immediate decision** for ADW use be made by the permittee and toxicity testing laboratory. The second is in the case where two of the most recent documented incidents of unacceptable site dilution water toxicity requires 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-New England
One Congress St., Suite 1100
Boston, MA 02114-2023

and

Manager
Water Technical Unit (SEW)
U.S. Environmental Protection Agency
One Congress Street, Suite 1100
Boston, MA 02114-2023

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

Method specific test conditions and TAC are to be followed and adhered to as specified in the method guidance document, EPA 821-R-02-013. 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.1. Use of Reference Toxicity Testing

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

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.

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.1.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.

V.2. For the *C. dubia* test, the determination of TAC and formal statistical analyses must be performed using only the first three broods produced.

V.3. Test treatments must include 5 effluent concentrations and a dilution water control. An additional test treatment, at the permitted effluent concentration (% effluent), is required if it is not included in the dilution series.

VI. CHEMICAL ANALYSIS

As part of each toxicity test's daily renewal procedure, pH, specific conductance, dissolved oxygen (DO) and temperature must be measured at the beginning and end of each 24-hour period in each test treatment and the control(s).

The additional analysis that must be performed under this protocol is as specified and noted in the table below.

Parameter	Effluent	Receiving Water	ML (mg/l)
Hardness[1, 4]	x	x	0.5
Total Residual Chlorine (TRC)[2, 3, 4]	x		0.02
Alkalinity[4]	x	x	2.0
pH[4]	x	x	--
Specific Conductance[4]	x	x	--
Total Solids [6]	x		--
Total Dissolved Solids [6]	x		--
Ammonia[4]	x	x	0.1
Total Organic Carbon [6]	x	x	0.5
Total Metals [5]			
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:

1. 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)

2. 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
- USEPA 1983. Manual of Methods Analysis of Water and Wastes
  -Method 330.5

3. Required to be performed on the sample used for WET testing prior to its use for toxicity testing
4. Analysis is to be performed on samples and/or receiving water, as designated in the table above, from all three sampling events.
5. Analysis is to be performed on the initial sample(s) only unless the situation arises as stated in Section III, paragraph 4
6. Analysis to be performed on initial samples only

VII. TOXICITY TEST DATA ANALYSIS AND REVIEW

A. Test Review

1. Concentration / Response Relationship

A concentration/response relationship evaluation is required for test endpoint determinations from both Hypothesis Testing and Point Estimate techniques. The test report is to include documentation of this evaluation in support of the endpoint values reported. The dose-response review must be performed as required in Section 10.2.6 of EPA-821-R-02-013. Guidance for this review can be found at http://www.epa.gov/waterscience/methods/wet/pdf/wetguide.pdf . In most cases, the review will result in one of the following three conclusions: (1) Results are reliable and reportable; (2) Results are anomalous and require explanation; or (3) Results are inconclusive and a retest with fresh samples is required.

2. Test Variability (Test Sensitivity)

This review step is separate from the determination of whether a test meets or does not meet TAC. Within test variability is to be examined for the purpose of evaluating test sensitivity. This evaluation is to be performed for the sub-lethal hypothesis testing endpoints reproduction and growth as required by the permit. The test report is to include documentation of this evaluation to support that the endpoint values reported resulted from a toxicity test of adequate sensitivity. This evaluation must be performed as required in Section 10.2.8 of EPA-821-R-02-013.

To determine the adequacy of test sensitivity, USEPA requires the calculation of test percent minimum significant difference (PMSD) values. In cases where NOEC determinations are made based on a non-parametric technique, calculation of a test PMSD value**,** for the sole purpose of assessing test sensitivity, shall be calculated using a comparable parametric statistical analysis technique. The calculated test PMSD is then compared to the upper and lower PMSD bounds shown for freshwater tests in Section 10.2.8.3, p. 52, Table 6 of EPA-821-R-02-013. The comparison will yield one of the following determinations.

- The test PMSD exceeds the PMSD upper bound test variability criterion in Table 6, the test results are considered highly variable and the test may not be sensitive enough to determine the presence of toxicity at the permit limit concentration (PLC). If the test results indicate that the discharge is not toxic at the PLC, then the test is considered insufficiently sensitive and must be repeated within 30 days of the initial test completion using fresh samples. If the test results indicate that the discharge is toxic at the PLC, the test is considered acceptable and does not have to be repeated.

- The test PMSD falls below the PMSD lower bound test variability criterion in Table 6, the test is determined to be very sensitive. In order to determine which treatment(s) are statistically significant and which are not, for the purpose of reporting a NOEC, the relative percent difference (RPD) between the control and each treatment must be calculated and compared to the lower PMSD boundary. See *Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the NPDES Program*, EPA 833-R-00-003, June 2002, Section 6.4.2. The following link: Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the NPDES Program can be used to locate the USEPA website containing this document. If the RPD for a treatment falls below the PMSD lower bound, the difference is considered statistically insignificant. If the RPD for a treatment is greater that the PMSD lower bound, then the treatment is considered statistically significant.

- The test PMSD falls within the PMSD upper and lower bounds in Table 6, the sub-lethal test endpoint values shall be reported as is.

B. Statistical Analysis

1. General - Recommended Statistical Analysis Method

Refer to general data analysis flowchart, EPA 821-R-02-013, page 43

For discussion on Hypothesis Testing, refer to EPA 821-R-02-013, Section 9.6

For discussion on Point Estimation Techniques, refer to EPA 821-R-02-013, Section 9.7

2. *Pimephales promelas*

Refer to survival hypothesis testing analysis flowchart, EPA 821-R-02-013, page 79

Refer to survival point estimate techniques flowchart, EPA 821-R-02-013, page 80

Refer to growth data statistical analysis flowchart, EPA 821-R-02-013, page 92

3. *Ceriodaphnia dubia*

Refer to survival data testing flowchart, EPA 821-R-02-013, page 168

Refer to reproduction data testing flowchart, EPA 821-R-02-013, page 173

VIII. TOXICITY TEST REPORTING

A report of results must include the following:

- Test summary sheets (2007 DMR Attachment F) 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
  - Test sensitivity evaluation results (test PMSD for growth and reproduction)
  - Permit limit and toxicity test results
  - Summary of test sensitivity and concentration response evaluation

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 limits (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
- Any further discussion of reported test results, statistical analysis and concentration-response relationship and test sensitivity review per species per endpoint