MARINE CHRONIC

TOXICITY TEST PROCEDURE AND PROTOCOL

I. GENERAL REQUIREMENTS

The permittee shall be responsible for the conduct of acceptable silverside chronic and sea urchin chronic toxicity tests in accordance with the appropriate test protocols described below:

- Inland Silverside (Menidia beryllina) Larval Growth and Survival Test
- Sea Urchin (Arbacia punctulata) 1 Hour Fertilization Test

Chronic toxicity 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:

https://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods

The permittee shall also meet the sampling, analysis and reporting requirements included in this protocol. Where 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 AND USE

A total of three fresh samples of effluent and receiving water are required for initiation and subsequent renewals of a marine, 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 fresh 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 fresh 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.

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.

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. For TRC analysis performed on site the results must be included on the chain of custody (COC) presented to WET laboratory. For the purpose of sample preparation, i.e. eliminating chlorine prior to toxicity testing, if called for by the permit, TRC analysis may also be performed by the toxicity testing laboratory and the samples must be dechlorinated, as necessary, using sodium thiosulfate prior to sample use for toxicity testing. According to 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 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.

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 oxidants (as per 40 CFR Part 122.21).

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 test acceptability criteria (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 the receiving water diluent is found to be, or suspected to be toxic or unreliable, an alternatedilution water (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 with supporting documentation must be sent electronically to the NPDES Applications Coordinator in EPA Water Division (WD) at the following email address:

R1NPDESReporting@epa.gov

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 EPA Region 1 website at https://www.epa.gov/aboutepa/epa-region-1-new-england (click on NPDES, EPA Permit Attachments, Self-Implementing Alternate Dilution Water Guidance) for important details on alternate dilution water substitution requests.

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.

V. TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

EPA New England requires that if a reference toxicant test was being performed concurrently with an effluent or receiving water test and fails, both tests must be repeated.

The following tables summarize the accepted Menidia and Arbacia toxicity test conditions and test acceptability criteria:

EPA NEW ENGLAND RECOMMENDED TEST CONDITIONS FOR THE SEA URCHIN, ARBACIA PUNCTULATA, FERTILIZATION TEST[1]

1. Test type	Static, non-renewal
2. Salinity	30 o/oo ± 2 o/oo by adding dry ocean salts
3. Temperature	20 ± 1°C temperature must not deviate by more than 3°C during test
4. Light quality	Ambient laboratory illumination
5. Light intensity	10-20 uE/m^2/s, or 50-100 ft-c (Ambient Laboratory Levels)
6. Test vessel size	Disposal (glass) liquid scintillation vials (20 ml capacity), presoaked in control water
7. Test solution volume	5 ml
8. Number of sea urchins	Pooled sperm from four males and pooled eggs from four females are used per test
9. Number of egg and sperm cells	About 2000 eggs per chamber and 5,000,000 sperm cells per vial
10. Number of replicate chambers	4 per treatment
11. Dilution water	Uncontaminated source of natural seawater or deionized water mixed with artificial sea salts
12. Dilution factor	Approximately 0.5, must bracket the permitted RWC
13. Test duration	1 hour and 20 minutes
14. Effects measured	Fertilization of sea urchin eggs
15. Number of treatments per test[2]	5 and a control. (receiving water and laboratory water control) An additional dilution at the permitted effluent concentration (% effluent) is required.

16. Acceptability of test	70% - 90% egg fertilization in all controls. Minimum of 70% fertilization in dilution water control. Effluent concentrations exhibiting greater than 70% fertilization, flagged as statistically significantly different from the controls, will not be considered statistically different from the controls for NOEC reporting.
17. Sampling requirements	For on-site tests, samples are to be 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.
18. Sample volume required	Minimum 1 liter

Footnotes:

[1] Adapted from EPA 821-R-02-014

EPA NEW ENGLAND RECOMMENDED TEST CONDITIONS FOR THE INLAND SILVERSIDE, MENIDIA BERYLLINA, GROWTH AND SURVIVAL TEST[1]

1. Test type	Static, renewal
2. Salinity	5 o/oo to 32 o/oo +/- 2 o/oo of the selected salinity by adding artificial sea salts
3. Temperature	25 ± 1°C, temperature must not deviate by more than 3°C during test
4. Light quality	Ambient laboratory light
5. Light intensity	10-20 uE/m^2/s, or 50-100 ft-C (Ambient Laboratory Levels)
6. Photoperiod	16 hr light, 8 hr darkness
7. Test vessel size	600 - 1000 mL beakers or equivalent (glass test chambers should be used)
8. Test solution volume	500-750 mL/replicate loading and DO restrictions must be met)
9. Renewal of test solutions	Daily using most recently collected sample
10. Age of test organisms	Seven to eleven days post hatch; 24 hr range in age
11. Larvae/test chamber	15 (minimum of 10)
12. Number of replicate chambers	4 per treatment
13. Source of food	Newly hatched and rinsed Artemia nauplii less than 24 hr old
14. Feeding regime	Feed once a day 0.10 g wet wt Artemia nauplii per replicate on days 0 – 2 feed 0.15 g wet wt Artemia nauplii per replicate on days 3-6
15. Cleaning	Siphon daily, immediately before test solution renewal and feeding
16. Aeration[2]	None
17. Dilution water	Uncontaminated source of natural seawater; or deionized water mixed with artificial sea salts

18. Effluent concentrations	5 and a control (receiving water and laboratory water control) An additional dilution at the permitted effluent concentration (% effluent) is required
19. Dilution factor	$\geq$ 0.5, must bracket the permitted RWC
20. Test duration	7 days
21. Effects measured	Survival and growth (weight)
22. Acceptability of test	The average survival of dilution water control larvae is a minimum of 80%, and the average dry wt of unpreserved control larvae is a minimum of 0.5 mg, or the average dry wt of preserved control larvae is a minimum of 0.43 mg if preserved not more than 7 days in 4% formalin or 70% ethanol
23. Sampling requirements	For on-site tests, samples are collected daily and used within 24 hours of the time they are removed from the sampling device. For off-site tests, samples must be first used within 36 hours of collection.
24. Sample Volume Required	Minimum of 6 liters/day.

Footnotes:

[1] Adapted from EPA 821-R-02-014

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

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. $\geq$3 standard deviations for IC25s values and $\geq$ two concentration intervals for NOECs, and even though the primary test meets TAC, the primary test will be considered unacceptable and must be repeated.

VI. CHEMICAL ANALYSIS

The toxicity test requires measurement of pH, salinity, and temperature at the beginning and end of each 24 hour period in each dilution and controls for both daily test renewal and waste. The following chemical analyses shall be performed for each initial sample as well as any renewal samples, if necessary pursuant to the requirement of Part III above.

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 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-02-014. Guidance for this review can be found at:
https://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods

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 endpoint growth for *Menidia beryllina* 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-02-014.

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 marine tests in Section 10.2.8.3, p. 54, Table 6 of EPA-821-R-02-014. 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-003, June 2000, Section 6.4.2. This document can be located under Guidance Documents at the following USEPA website location:

https://www.epa.gov/aboutepa/epa-region-1-new-england
(click on NPDES, EPA Permit Attachments)

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-02-014, page 45

For discussion on Hypothesis Testing, refer to EPA 821-R-02-014, Section 9.6

For discussion on Point Estimation Techniques, refer to EPA 821-R-02-014, Section 9.7

2. *Menidia beryllina*

Refer to survival hypothesis testing analysis flowchart, EPA 821-R-02-014, page 181

Refer to survival point estimate techniques flowchart, EPA 821-R-02-013, page 182

Refer to growth data statistical analysis flowchart, EPA 821-R-02-014, page 193

3. *Arbacia punctulata*

Refer to fertilization data testing flowchart, EPA 821-R-02-014, page 312

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
  - Test sensitivity evaluation results (test PMSD for growth)
  - Permit limit and toxicity test results
  - Summary of test sensitivity and concentration response evaluation

Please note: The NPDES Permit Program Instructions for the Discharge Monitoring Report Forms (DMRs) are available on EPA's website at: www.epa.gov/compliance/discharge-monitoring-reports-avoiding-common-mistakes

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; and
- Any further discussion of reported test results, statistical analysis and concentration-response relationship and test sensitivity review.