MARINE CHRONIC
TOXICITY TEST PROCEDURE AND PROTOCOL

I. GENERAL REQUIREMENTS

The permittee shall conduct acceptable silverside chronic (and modified acute) 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 and acute toxicity data shall be reported as outlined in Section VIII. The chronic Menidia test can be used to calculate an LC50 at the end of 48 hours of exposure when both an acute (LC50) and a chronic (C-NOEC) test is specified in the permit.

II. METHODS

Methods to follow are those recommended by EPA in:

Klemm, D.J. et al. Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters To Marine and Estuarine Organisms, Second Edition. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, July 1994, EPA/600/4-91/003. https://www.epa.gov/cwa-methods/whole-effluent-toxicity-methods

Any exceptions are stated herein.

III. SAMPLE COLLECTION

For each sampling event involving the Menidia beryllina, three discharge samples shall be collected. Fresh samples are necessary for Days 1, 3, and 5 (see Section V. for holding times). A single sample is necessary for the Arbacia punctulata test. The sample shall be analyzed chemically (see Section VI). The initial sample (Day 1) is used to start the tests, and for test solution renewal on Day 2. The second sample is collected for use at the start of Day 3, and for renewal on Day 4. The third sample is used on Days 5, 6, and 7. The initial (Day 1) sample will be analyzed chemically (see Section VI). Day 3 and 5

renewal samples will be held until test completion. If either the Day 3 or 5 renewal sample is of sufficient potency to cause lethality to 50 percent or more test organisms in any of the dilutions for either species, then a chemical analysis shall be performed on the appropriate sample(s) as well.

Aliquots shall be split from the sample, containerized and preserved (as per 40 CFR Part 136) for the chemical and physical analyses. The remaining sample shall be 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 oxidants (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 mg/L chlorine. A thiosulfate control (maximum amount of thiosulfate in lab control or receiving water) should also be run.

All samples held overnight shall be refrigerated at 4°C.

IV. DILUTION WATER

Grab samples of receiving water used for chronic toxicity testing shall be collected from one or several distances away from the discharge. It may be necessary to test receiving water at several distances in a separate chronic test to determine the extent of the zone of toxicity. Avoid collecting near areas of obvious road or agricultural runoff, storm sewers or other point source discharges. An additional control (0% effluent) of a standard laboratory water of known quality shall 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 conductivity, salinity, total suspended solids, organic carbon, and pH 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 alternative dilution water should be mailed with supporting documentation to the following address:

Director
Office of Ecosystem Protection
U. S. Environmental Protection Agency-New England
JFK Federal Building (CAA)
Boston, MA 02203

It may prove beneficial to the permittee 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 AND TEST ACCEPTABILITY CRITERIA

EPA New England requires that tests be performed using four replicates of each control and effluent concentration because the on-parametric statistical tests cannot be used with data from fewer replicates. Also, 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
4. Light quality	Ambient laboratory light during test preparation
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 per chamber	About 2000 eggs and 5,000,000 sperm cells per vial
10. Number of replicate chambers per treatment	4
11. Dilution water	Uncontaminated source of natural seawater or deionized water mixed with artificial sea salts
12. Dilution factor	Approximately 0.5
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. An additional dilution at the permitted effluent concentration (% effluent) is required.
16.	Acceptability of test	Minimum of 70% fertilization in controls. 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/600/4-91/003, July 1994.

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

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 by adding artificial sea salts
3.	Temperature	25 ± 1°C
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[3]	5 and a control. An additional dilution at the permitted effluent concentration (% effluent) is required.
19.	Dilution factor	$\geq$ 0.5
20.	Test duration	7 days
21.	Effects measured	Survival and growth (weight)
22.	Acceptability of test	The average survival of 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/600/4-91/003, July 1994.

[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.

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

VI. CHEMICAL ANALYSIS

As part of each daily renewal of the Menidia test, pH, dissolved oxygen, salinity, and temperature must be measured at the beginning and end of each 24 hour period in each dilution and in the controls. It must also be done at the start of the Arbacia test. The following chemical analyses shall be performed for each sampling event.

Parameter	Effluent	Diluent	Minimum Quantification Level(mg/L)
pH	x	x	---
Salinity	x	x	PPT(o/oo)
Total Residual Oxidants[*1]	x	x	0.05
Total Solids and Suspended Solids	x	x	

Ammonia	x	x	
	0.1		
Total Organic Carbon	x	x	
	0.5		
Total Metals			
Cd	x		0.001
Cr	x		0.005
Pb	x		0.005
Cu	x		0.0025
Zn	x		0.0025
Ni	x		0.004
Al	x		0.02

Superscripts:

[*1] Total Residual Oxidants
Either of the following methods from the 18th Edition of the APHA (1992) Standard Methods for the Examination of Water and Wastewater must be used for these analyses:

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

or use USEPA Manual of Methods Analysis of Water or Wastes, Method 330.5.

VII. TOXICITY TEST DATA ANALYSIS

LC50 Median Lethal Concentration (Determined at 48 Hours)

Methods of Estimation:

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

See flow chart on page 56 of EPA/600/4-91/003 for appropriate point estimation method to use on a given data set.

Chronic No Observed Effect Concentration (C-NOEC)

Methods of Estimation:

- Dunnett's Procedure
- Bonferroni's T-Test
- Steel's Many-One Rank Test
- Wilcoxin Rank Sum Test

Reference flow charts on pages 191, 192, and 321 of EPA/600/4-91/003 for the appropriate method to use on a given data set.

In the case of two tested concentrations causing adverse effects but an intermediate concentration not causing a statistically significant effect, report the C-NOEC as the lowest concentration where there is no observable effect. The definition of NOEC in the EPA Technical Support Document only applies to linear dose-response data.

VIII. TOXICITY TEST REPORTING

A report of 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; and
- 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.