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:


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:
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. **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/m²/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

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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.
<table>
<thead>
<tr>
<th></th>
<th>EPA NEW ENGLAND RECOMMENDED TEST CONDITIONS FOR THE INLAND SILVERSIDE, MENIDIA BERYLLINA, GROWTH AND SURVIVAL TEST¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Test type</td>
</tr>
<tr>
<td>2.</td>
<td>Salinity</td>
</tr>
<tr>
<td>3.</td>
<td>Temperature</td>
</tr>
<tr>
<td>4.</td>
<td>Light quality</td>
</tr>
<tr>
<td>5.</td>
<td>Light intensity</td>
</tr>
<tr>
<td>6.</td>
<td>Photoperiod</td>
</tr>
<tr>
<td>7.</td>
<td>Test vessel size</td>
</tr>
<tr>
<td>8.</td>
<td>Test solution volume</td>
</tr>
<tr>
<td>9.</td>
<td>Renewal of test solutions</td>
</tr>
<tr>
<td>10.</td>
<td>Age of test organisms</td>
</tr>
<tr>
<td>11.</td>
<td>Larvae/test chamber</td>
</tr>
<tr>
<td>12.</td>
<td>Number of replicate chambers</td>
</tr>
<tr>
<td>13.</td>
<td>Source of food</td>
</tr>
<tr>
<td>14.</td>
<td>Feeding regime</td>
</tr>
<tr>
<td>15.</td>
<td>Cleaning</td>
</tr>
</tbody>
</table>

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16. Aeration²  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.

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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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Effluent</th>
<th>Diluent Level (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Salinity</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Total Residual Oxidants&lt;sup&gt;1&lt;/sup&gt;</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Total Solids and Suspended Solids</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Ammonia</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Total Organic Carbon</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

**Total Metals**

<table>
<thead>
<tr>
<th>Element</th>
<th>Effluent</th>
<th>Level (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>x</td>
<td>0.001</td>
</tr>
<tr>
<td>Cr</td>
<td>x</td>
<td>0.005</td>
</tr>
<tr>
<td>Pb</td>
<td>x</td>
<td>0.005</td>
</tr>
<tr>
<td>Cu</td>
<td>x</td>
<td>0.0025</td>
</tr>
<tr>
<td>Zn</td>
<td>x</td>
<td>0.0025</td>
</tr>
<tr>
<td>Ni</td>
<td>x</td>
<td>0.004</td>
</tr>
<tr>
<td>Al</td>
<td>x</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Superscripts:**

<sup>1</sup> 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.

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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.)

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• Raw data and bench sheets.

• Provide a description of dechlorination procedures (as applicable).

• Any other observations or test conditions affecting test outcome.