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Proposed Changes to Whole Effluent Toxicity Method Manuals

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I. Introduction

In 1995, the U.S. Environmental Protection Agency (EPA) published a final rule standardizing 17 whole effluent toxicity (WET) test methods for use in National Pollutant Discharge Elimination System (NPDES) monitoring [60 FR 53529; October 16, 1995]. These WET test methods measure the toxicity of effluents and receiving waters to freshwater, marine, and estuarine organisms. The approved methods include acute toxicity methods and short-term methods for estimating chronic toxicity. Acute methods generally use death of the test organisms during 24-96 hour exposure durations as the measured effect of an effluent or receiving water. The short-term methods for estimating chronic toxicity use longer durations of exposure (up to nine days) to ascertain the adverse effects of an effluent or receiving water on survival, growth, and/or reproduction of the organisms. In this document, the short-term methods for estimating chronic toxicity will be referred to as chronic methods for ease of notation. Standardized test procedures for conducting the approved WET test methods are published in the following three test method manuals (the WET method manuals):

- U.S. Environmental Protection Agency. 1993. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 4th ed. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH. (the acute method manual)
- U.S. Environmental Protection Agency. 1994a. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, 3rd ed. EPA/600/4-91/002. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH. (the freshwater chronic method manual)
- U.S. Environmental Protection Agency. 1994b. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*, 2nd ed. EPA/600/4-91/003. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH. (the marine chronic method manual)

Since the publishing of these method manuals, EPA has corrected minor errors and omissions by providing the following errata and addenda documents.

- U.S. Environmental Protection Agency. 1996. *Addenda for Acute Manual*. In U.S. Environmental Protection Agency, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 4th ed. EPA/600/4-90/027F. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH.
- U.S. Environmental Protection Agency. 1999. *Errata for Effluent and Receiving Water Toxicity Test Manuals: Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms; Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms; and Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms.* January 1999. EPA/600/R-98/182. U.S. Environmental Protection Agency, Office of Research and Development, Duluth, MN.

EPA anticipates proposing to revise each of the WET method manuals to incorporate the above errata and addenda, update the methods, provide additional minor corrections and clarifications, and address

specific stakeholder concerns. When EPA takes final action on this proposal, the Agency intends to incorporate the proposed modifications into the text of new editions of each of the WET method manuals. This document details the proposed changes to the WET method manuals in redline and strikeout text formatting. The proposed changes and the method manual sections affected are summarized in Table 1.

Table 1. Summary of Proposed WET Method Manual Changes.

Chapter	Summary of Change	Method Manual Sections Affected		
		Acute Manual	**Freshwater Chronic Manual**	**Marine Chronic Manual**
II. Minor Corrections and Clarifications	correct minor technical errors and omissions and add references cited in the proposed changes	Section 5, 8, & References	Section 5, 6, 8, 11, 13, 14, & References	Section 5, 6, 8, 11, 12, 13, 14, 15, 16, & References
III. Precision	update method precision statements (including tables of CVs) with data from recent EPA studies	Section 4	Section 4, 11, 13, 14	Section 4, 11, 13, 14, 16
IV. Blocking by Known Parentage	require blocking by known parentage in the *Ceriodaphnia dubia* Survival and Reproduction Test		Section 13	
V. pH Drift	add procedures for control of pH drift during testing	Section 9	Section 11, 12, 13	Section 11, 12, 13, 14
VI. Concentration-response Relationships	incorporate required review procedures for evaluating concentration-response relationships	Section 12	Section 10	Section 10
VII. Nominal Error Rates	clarify allowable nominal error rate adjustments		Section 9	Section 9
VIII. Confidence Intervals	clarify limitations in the generation of confidence intervals	Section 11	Section 9	Section 9
IX. Dilution Series	add guidance on dilution series selection	Section 9	Section 8	Section 8
X. Dilution Water	clarify dilution water acceptability	Section 7	Section 7	Section 7
XI. Pathogen Interference	add guidance on controlling pathogen interference in the Fathead Minnow Larval Survival and Growth Test		Section 11	
XII. *Selenastrum capricornutum* Growth Test Method	recommend use of EDTA		Section 14	
XIII. *Mysidopsis bahia* Survival, Growth, and Fecundity Test Method	add guidance to improve success of the fecundity endpoint			Section 14
XIV. *Holmesimysis costata* Acute Test Method	add acute method for *Holmesimysis costata* including new table of test conditions and supplementary information in Appendix A.3	Section 6, 9, App. A.3, and B		
XV. Percent Minimum Significant Difference (PMSD)	add required application of upper and lower PMSD bounds for the *Ceriodaphnia dubia* Survival and Reproduction Test; Fathead Minnow Larval Survival and Growth Test; *Mysidopsis bahia* Survival, Growth, and Fecundity Test; and Inland Silverside Larval Survival and Growth Test		Section 11 and 13	Section 13 and 14

II. Minor Corrections and Clarifications

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Replace Subsection 5.1.4.1 on page 21 of the acute method manual with the following.

5.1.4.1 A ~~deionizing system~~ good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, ~~laboratory grade water should~~ must be ~~provided with~~ available in the laboratory and in sufficient ~~capacity~~ quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a CULLIG~~E~~AN®, CONTINENTAL®, or equivalent mixed-bed water treatment system.

2. Replace Subsection 8.5.4 on page 43 of the acute method manual with the following.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of ~~the~~ each grab or composite sample ~~in test initiation~~ must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the sample to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e), must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, ~~the original~~ each grab or composite sample may also be used to prepare test solutions for renewal at ~~24 h, 48 h, and/or 72 h after test initiation~~ 24 h and/or 48 h after first use, if stored at 4˚C, with minimum head space, as described in Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

3. Insert the following references into the Cited References section on page 121 of the acute method manual.

Casarett, L.J. and J. Doull. 1975. Toxicology: the basic science of poisons. Macmillan Publishing Co., New York.

Martin, M., J.W. Hunt, B.S. Anderson, S.L. Turpen, and F.H. Palmer. 1989. Experimental evaluation of the mysid *Holmesimysis costata* as a test organism for effluent toxicity testing. Environ. Toxicol. Chem. 8: 1003-1012.

Price, W.W., R.W. Heard, and L. Stuck. 1994. Observations on the genus *Mysidopsis* Sars, 1864 with the designation of a new genus, *Americamysis*, and the descriptions of *Americamysis alleni* and

Americamysis stucki (Peracarida: Mysidacea: Mysidae), from the Gulf of Mexico. Proc. Biol. Soc. Wash. 107: 680-698.

USEPA. 2000a. Method guidance and recommendations for whole effluent toxicity (WET) testing (40 CFR Part 136). Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-00/004.

USEPA. 2000b. Understanding and accounting for method variability in whole effluent toxicity applications under the national pollutant discharge elimination system program. Office of Wastewater Management, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/833/R-00/003.

USEPA. 2001a. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 1. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

USEPA. 2001b. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 2: Appendix. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Replace Subsection 5.4.2.1 on page 23 of the freshwater chronic method manual with the following.

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, ~~laboratory grade water, should~~ must be available in the laboratory and in sufficient ~~capacity~~ quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a Culligan®, Continental®, or equivalent mixed-bed water treatment system.

2. Replace Subsection 6.1.4 on page 26 of the freshwater chronic method manual with the following.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive, or more sensitive, than the species recommended in Sub~~S~~section 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the recommended species in ~~SubSection 6.2.6~~ Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

3. Replace Subsections 6.5.7 and 6.5.8 on page 29 of the freshwater chronic method manual with the following.

~~6.5.7 Fish should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish (5-10%) mortality during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Fish in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).~~

6.5.~~8~~7 A daily record of feeding, behavioral observations, and mortality should be maintained.

4. Replace Subsection 8.5.4 on page 38 of the freshwater chronic method manual with the following.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of ~~the~~ each grab or composite sample ~~in test initiation~~ must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the sample to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e), must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, ~~the original~~ each grab or composite sample may also be used to prepare test solutions for renewal at 24 h and/or 48 hr after ~~test initiation~~ first use, if stored at 4˚C, with minimum head space, as described in Sub~~S~~section 8.5. Guidance for determining the persistence of the sample is provided in Sub~~S~~section 8.7.

5. Replace Subsection 8.8.2 on page 39 of the freshwater chronic method manual with the following.

8.8.2 With the daphnids, *Ceriodaphnia dubia*, and fathead minnow, *Pimephales promelas*, tests, effluent and receiving waters should ~~must~~ be filtered through a 60-µm plankton net to remove indigenous organisms that may attack or be confused with test organisms (see the daphnid, *Ceriodaphnia dubia* test for more details). Receiving waters used in green alga, *Selenastrum capricornutum*, toxicity tests must be filtered through a 0.45-µm pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm mesh openings ~~holes~~ to remove debris and/or break up large floating or suspended solids. Because filtration may increase the dissolved oxygen (DO) in the effluent, the DO should be checked both before and after filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution**: filtration may remove some toxicity.

6. Replace item 23 in Table 1 (continued) on page 80 of the freshwater chronic method manual with the following.

23. Sampling requirements:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device; For off-site tests, a minimum of three samples collected on days one, three and five with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, and Subsection 8.5.4)

7. Replace Table 14 on page 100 of the freshwater chronic method manual with the following.

TABLE 14. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4734	0.352	$X^{(20)} - X^{(1)}$
2	0.3211	0.303	$X^{(19)} - X^{(2)}$
3	0.2565	~~0.131~~0.181	$X^{(18)} - X^{(3)}$
4	0.2085	0.106	$X^{(17)} - X^{(4)}$
5	0.1686	0.105	$X^{(16)} - X^{(5)}$
6	0.1334	0.065	$X^{(15)} - X^{(6)}$
7	0.1013	0.049	$X^{(14)} - X^{(7)}$
8	0.0711	0.031	$X^{(13)} - X^{(8)}$
9	0.0422	0.012	$X^{(12)} - X^{(9)}$
10	0.0140	0.008	$X^{(11)} - X^{(10)}$

8. Replace Subsections 13.13.3.6.3 and 13.13.3.6.4 on page 182 of the freshwater chronic method manual with the following.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(45)\ln(31.8) - 9 \sum_{i=1}^{P} \ln(S_i^2)]/1.04$$

= [45(~~3.5~~ 3.46) - 9(~~16.1~~ 16.061)]/1.04

= ~~12.6~~ 11.15/1.04

= ~~12.1~~ 10.72

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with four degrees of freedom, is 13.3. Since B = ~~12.1~~10.7 is less than the critical value of 13.3, conclude that the variances are not different.

9. Replace Subsection 14.6.16.1 on page 199 of the freshwater chronic method manual with the following.

14.6.16.1 *Selenastrum capricornutum*, a unicellular coccoid green alga is the test organism. The genus and species name of this organism was formally changed to *Pseudokirchneriella subcapitata* (Hindak, 1990), however, the method manual will continue to refer to *Selenastrum capricornutum* to maintain consistency with previous versions of the method.

10. Replace item 19 in Table 3 (continued) on page 211 of the freshwater chronic method manual with the following.

19. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, and Subsection 8.5.4)

11. Insert the following references into the Cited References section on page 229 of the freshwater chronic method manual.

Casarett, L.J. and J. Doull. 1975. Toxicology: the basic science of poisons. Macmillan Publishing Co., New York.

Downey, P.J.,K. Fleming, R. Guinn, N. Chapman, P. Varner, J. Cooney. 2000. Sporadic mortality in chronic toxicity tests using *Pimephales promelas* (Rafinesque): cases of characterization and control. Environ. Toxicol. Chem. 19(1): 248-255.

Geis, S., K. Fleming, A. Mager, and K. Schappe. 2000. Investigation of the pathogenic effect in whole effluent toxicity (WET) chronic fathead minnow tests. SETAC Abstract Book, 21st Annual Meeting, 12-16 November, 2000.

Hindak, F. 1990. Biologicke prace (Slovenskej Akademie Vied). 36: 209.

Mount, D.R. and D.I. Mount. 1992. A simple method of pH control for static and static renewal aquatic toxicity tests. Environ. Toxicol. Chem. 11: 609-614.

USEPA. 2000a. Method guidance and recommendations for whole effluent toxicity (WET) testing (40 CFR Part 136). Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-00/004.

USEPA. 2000b. Understanding and accounting for method variability in whole effluent toxicity applications under the national pollutant discharge elimination system program. Office of

Wastewater Management, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/833/R-00/003.

USEPA. 2001a. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 1. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

USEPA. 2001b. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 2: Appendix. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Replace Subsection 5.4.2.1 on page 25 of the marine chronic method manual with the following.

5.4.2.1 A good quality, laboratory grade deionized water, providing a resistance of 18 megaohm-cm, ~~laboratory grade water, should~~ must be available in the laboratory and ~~with~~ in sufficient ~~capacity~~ quantity for laboratory needs. Deionized water may be obtained from MILLIPORE® Milli-Q®, MILLIPORE QPAK™$_2$ or equivalent system. If large quantities of high quality deionized water are needed, it may be advisable to supply the laboratory grade water deionizer with preconditioned water from a Culligean®, Continental®, or equivalent mixed-bed water treatment system.

2. Replace Subsection 6.1.4 on page 27 of the marine chronic method manual with the following.

6.1.4 Some states have developed culturing and testing methods for indigenous species that may be as sensitive or more sensitive, than the species recommended in Subsection 6.1.3. However, USEPA allows the use of indigenous species only where state regulations require their use or prohibit importation of the species in ~~Section 6, Facilities, Equipment, and Supplies,~~ Subsection 6.1.3. Where state regulations prohibit importation of non-native fishes or use of the recommended test species, permission must be requested from the appropriate state agency prior to their use.

3. Replace Subsections 6.5.7 and 6.5.8 on page 30 of the marine chronic method manual with the following.

~~6.5.7 Fish should be observed carefully each day for signs of disease, stress, physical damage, and mortality. Dead and abnormal specimens should be removed as soon as observed. It is not uncommon to have some fish (5-10%) mortality during the first 48 h in a holding tank because of individuals that refuse to feed on artificial food and die of starvation. Fish in the holding tanks should generally be fed as in the cultures (see culturing methods in the respective methods).~~

6.5.~~8~~7 A daily record of feeding, behavioral observations, and mortality should be maintained.

4. Replace Subsection 8.5.4 on page 40 of the marine chronic method manual with the following.

8.5.4 Sample holding time begins when the last grab sample in a series is taken (i.e., when a series of four grab samples are taken over a 24-h period), or when a 24-h composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time (holding time) from sample collection to first use of ~~the~~ each grab or composite sample ~~in test initiation~~ must not exceed 36 h. EPA believes that 36 h is adequate time to deliver the sample to the laboratories performing the test in most cases. In the isolated cases, where the permittee can document that this delivery time cannot be met, the permitting authority can allow an option for on-site testing or a variance for an extension of shipped sample holding time. The request for a variance in sample holding time, directed to the USEPA Regional Administrator under 40 CFR 136.3(e), must include supportive data which show that the toxicity of the effluent sample is not reduced (e.g., because of volatilization and/or sorption of toxics on the sample container surfaces) by extending the holding time beyond more than 36 h. However, in no case should more than 72 h elapse between collection and first use of the sample. In static-renewal tests, ~~the original~~ each grab or composite sample may also be used to prepare test solutions for renewal at 24 h and/or 48 h after ~~test initiation~~ first use, if stored at 4˚C, with minimum head space, as described in ~~Paragraph~~ Subsection 8.5. Guidance for determining the persistence of the sample is provided in Subsection 8.7.

5. Replace Subsection 8.8.3 on page 41 of the marine chronic method manual with the following.

8.8.3 It may be necessary to first coarse-filter samples through a NYLON® sieve having 2 to 4 mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples ~~must~~ should be filtered through a sieve with 60-µm mesh openings. Since filtering may increase the dissolved oxygen (DO) in an effluent, the DO should be checked both before and after ~~determined prior to~~ filtering. Low dissolved oxygen concentrations will indicate a potential problem in performing the test. **Caution**: filtration may remove some toxicity.

6. Replace Subsection 11.13.3.5.1 on page 102 of the marine chronic method manual with the following.

11.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 14.

7. Replace Subsections 11.13.3.6.3 and 11.13.3.6.4 on page 106 of the marine chronic method manual with the following.

11.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(\text{~~0.0137~~ } 0.0187) - 3\sum_{i=1}^{P}\ln(S_i^2)\ ]/1.139$$

$$= [12(\text{~~-4.290~~ } 3.979) - 3(\text{~~-18.960~~}18.876)]/1.139$$

= ~~5.396~~ 8.882/1.139

= ~~4.737~~ 7.798

11.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is 11.345. Since B = ~~4.737~~ 7.798 is less than the critical value of 11.345, conclude that the variances are not different.

8. Replace Subsection 12.5.2 on page 125 of the marine chronic method manual with the following.

12.5.2 Sheepshead minnow culture unit -- see Subsection ~~6.13~~ 12.6.12 below. To perform toxicity tests on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from an in-house sheepshead minnow culture unit. If necessary, embryos can be obtained from outside sources if shipped in well oxygenated water in insulated containers.

9. Replace Subsection 12.5.3 on page 125 of the marine chronic method manual with the following.

12.5.3 Brine shrimp, *Artemia*, culture unit -- for feeding sheepshead minnow larvae in the continuous culture unit (see Subsection ~~6.12~~ 12.6.11 below).

10. Replace Subsection 12.6.10.1 on page 127 of the marine chronic method manual with the following.

12.6.10.1 Saline test and dilution water -- The salinity of the test water must be in the range of 5 to 32‰. The salinity should vary no more than ±2‰~~t~~ among chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of the water should be similar.

11. Replace Subsection 12.6.10.2 on page 127 of the marine chronic method manual with the following.

12.6.10.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sheepshead minnow embryos to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. ~~If~~ In addition, it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities -- a hypersaline brine derived from natural seawater or artificial sea salts.

12. Replace Note to Figure 1 on page 128 of the marine chronic method manual with the following.

Note: Final endpoint for this test is total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) (see Subsection 12.10.~~8~~ 9 and 12.13).

13. Replace Subsection 12.10.7.1 on page 141 of the marine chronic method manual with the following.

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the tank causes a problem.

14. Replace Subsection 13.10.9.4 on page 186 of the marine chronic method manual with the following.

13.10.9.4 Immediately prior to drying, the preserved larvae are in distilled water. The rinsed larvae from each test chamber are transferred, using forceps, to a tared weighing pans and dried at 60˚C for 24 h, or at 105˚C for a minimum of 6 h. Immediately upon removal from the drying oven, the weighing pans are placed in a desiccator to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing pans containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate. Record (Figure 4) the weights. Divide the dry weight by the number of original larvae per replicate to determine the average dry weight, and record (Figures 4 and 5) on the data sheets. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria (see Subsection 13.11). Complete the summary data sheet (Figure 5) after calculating the average measurements and statistically analyzing the dry weights and percent survival for the entire test. Average weights should be expressed to the nearest 0.001 mg.

15. Replace Table 12 on page 205 of the marine chronic method manual with the following.

TABLE 12. INLAND SILVERSIDE, *MENIDIA BERYLLINA*, GROWTH DATA

		Effluent Concentration %				
Replicate	Control	6.25	12.5	25.0	50.0	100.0
A	0.751	0.737	0.722	0.196	-	-
B	0.849	0.922	0.285	0.312	-	-
C	0.907	0.927	0.718	~~1.079~~ 0.079	-	-
Mean ($\bar{Y}_i$)	0.836	~~0.859~~ 0.862	0.575	0.196	-	-
S_i^2	0.0062	~~0.0130~~ 0.0117	0.0631	0.0136	-	-
i	1	2	3	4	5	6

16. Replace Table 13 on page 205 of the marine chronic method manual with the following.

TABLE 13. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)		
Replicate	Control	6.25	12.5
A	-0.085	~~0.147~~ -0.125	~~0.00~~ 0.147
B	0.013	~~-0.290~~ 0.060	~~0.166~~ -0.290
C	0.071	~~0.143~~ 0.065	~~-0.117~~ 0.143

17. Replace Subsection 13.13.3.5.2 on page 207 of the marine chronic method manual with the following.

13.13.3.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

X= the overall mean of the centered observations

n = the total number of centered observations.

For this set of data, n = 9

$$\bar{X} = \frac{1}{9}(-0.002) = 0.000$$

D = ~~0.167~~ 0.162

18. Replace Table 14 on page 207 of the marine chronic method manual with the following.

TABLE 14. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.290	6	~~0.071~~ 0.065
2	~~-0.117~~ -0.125	7	~~0.116~~ 0.071
3	-0.085	8	0.143

4	~~0.000~~ 0.013	9	0.147
5	~~0.013~~ 0.060		

19. Replace Subsection 13.13.3.5.5 and Table 15 on page 208 of the marine chronic method manual with the following.

13.13.3.5.5 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 15. For this set of data:

$$W = \frac{1}{\text{~~0.1657~~ 0.162}}\left(\text{~~0.3997~~ 0.3800}\right)^2 = \text{~~0.964~~ 0.89}$$

TABLE 15. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.5888	0.437	$X^{(9)} - X^{(1)}$
2	0.3244	~~0.260~~ 0.268	$X^{(8)} - X^{(2)}$
3	0.1976	~~0.201~~ 0.156	$X^{(7)} - X^{(3)}$
4	0.0947	~~0.071~~ 0.052	$X^{(6)} - X^{(4)}$

20. Replace Subsections 13.13.3.6.3 and 13.13.3.6.4 on page 209 of the marine chronic method manual with the following.

13.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(6)\ln(\text{~~0.274~~ 0.027}) - 2\sum_{i=1}^{P}\ln(S_i^2)\ ] / \text{~~1.25~~ 1.222}$$

$$= [6(\text{~~-3.5972~~ -3.612}) - 2(\text{~~1n(0.0062)+1n(0.0130)+1n(0.0631)~~ -12.290})]/\text{~~1.25~~ 1.222}$$

$$= \text{~~[-26.583 - (-24.378)]~~ 2.909}/\text{~~1.25~~ 1.222}$$

$$= \text{~~2.236~~ 2.38}$$

13.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 2 degrees of freedom, is 9.210. Since B = ~~2.236~~ 2.38 is less than the critical value of 9.210, conclude that the variances are not different.

21. Replace Subsection 14.5.3 on page 223 of the marine chronic method manual with the following.

14.5.3 Mysid, *Mysidopsis bahia*, culture unit -- see Subsection ~~6~~ 14.6.13 below. This test requires a minimum of 240 7-day old (juvenile) mysids. It is preferable to obtain the test organisms from an in-house culture unit. If it is not feasible to culture mysids in-house, juveniles can be obtained from other sources, if shipped in well oxygenated saline water in insulated containers.

22. Replace Subsection 14.6.2 on page 225 of the marine chronic method manual with the following.

14.6.2 Data sheets (one set per test) -- for data recording (Figures ~~14, 15, and 16~~ 2, 7, and 8).

23. Replace Subsection 14.6.13, Test Organisms, on page 230 of the marine chronic method manual with the following.

14.6.13 TEST ORGANISMS, *Mysidopsis bahia* (see Rodgers *et al.*, 1986 and USEPA, 1993a for information on mysid ecology). The genus name of this organism was formally changed to *Americamysis* (Price *et al.*, 1994), however, the method manual will continue to refer to *Mysidopsis bahia* to maintain consistency with previous versions of the method.

24. Replace Subsection 14.6.13.2.1 on page 233 of the marine chronic method manual with the following.

14.6.13.2.1 The test is begun with 7-day-old juveniles. To have the test animals available and acclimated to test conditions at the start of the test, ~~they~~ gravid females must be obtained from the stock culture eight days in advance of the test. Whenever possible, brood stock should be obtained from cultures having similar salinity, temperature, light regime, etc., as are to be used in the toxicity test.

25. Replace Subsection 14.10.8.2.1 on page 239 of the marine chronic method manual with the following.

14.10.8.2.1 The number of live mysids are counted and recorded each day when the test solutions are renewed (Figure ~~2~~ 7). Dead animals and excess food should be removed with a pipette before test solutions are renewed.

26. Replace Subsection 14.12.1 on page 243 of the marine chronic method manual with the following.

14.12.1 The minimum requirements for an acceptable test are 80% survival and an average weight of at least 0.20 mg/surviving mysid in the controls. If fecundity in the controls is adequate (egg production by 50% of females), fecundity should be used as a criterion of effect in addition to survival and growth.

27. Replace Subsection 14.13.2.4 on page 252 of the marine chronic method manual with the following.

14.13.2.4 Probit Analysis (Finney, 1971; see Appendix ~~G~~ H) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined. If the data do not fit the Probit model, the Spearman-Karber method, the Trimmed Spearman-Karber method, or the Graphical method may be used (see Appendices ~~H~~ I-K).

28. Replace Subsection 14.13.2.6.6 on page 257 of the marine chronic method manual with the following.

14.13.2.6.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i\left(X^{(n-i+1)} - X^{(i)}\right)\right]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table ~~7~~ 8. For this data in this example:

$$W = \frac{1}{1.197}(1.0475)^2 = 0.9167$$

29. Replace Subsection 14.13.2.6.7 on page 258 of the marine chronic method manual with the following.

14.13.2.6.7 The decision rule for this test is to compare W as calculated in Subsection 14.13.2.6.~~5~~ 6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this set of data, the critical value at a significance level of 0.01 and n = 40 observations is 0.919. Since W = 0.9167 is less than the critical value, conclude that the data are not normally distributed.

30. Replace Subsections 14.13.3.6.3 and 14.13.3.6.4 on page 269 of the marine chronic method manual with the following.

14.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(28)\ln(0.00162) - 7\sum_{i=1}^{P}\ln(S_i^2)\ ] / 1.06$$

$$= [28(\text{~~-6.4315~~ -6.427}) - 7(\text{~~-25.9357~~ -25.9329})]/1.06$$

$$= [\text{~~-180.082~~ -179.973} - (\text{~~-181.5499~~ -181.530})]/1.06$$

$$= \text{~~1.385~~ 1.469}$$

14.13.3.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with three degrees of freedom, is ~~9.210~~ 11.34. Since B = ~~1.385~~ 1.469 is less than the critical value of 9.210, conclude that the variances are not different.

31. Replace Table 21 on page 281 of the marine chronic method manual with the following.

TABLE 21. MYSID, *MYSIDOPSIS BAHIA*, FECUNDITY DATA: PERCENT FEMALES WITH EGGS

		Test Concentration (ppb)			
	Replicate	Control	50.0	100.0	210.0
RAW	1	1.00	0.50	0.33	0.0
	2	1.00	0.33	0.50	0.0
	3	0.67	0.67	0.00	0.0
	4	1.00	-	0.50	0.0
	5	1.00	0.40	0.67	0.0
	6	0.80	0.50	0.00	0.0
	7	1.00	0.25	0.25	0.0
	8	1.00	0.33	-	0.0
ARC SINE TRANS-FORMED[1]	1	1.57	0.78	0.61	-
	2	1.57	0.61	0.78	-
	3	0.96	0.96	0.00	-
	4	1.57	-	0.78	-
	5	1.57	0.68	0.96	-
	6	1.12	0.78	0.00	-
	7	1.57	0.52	0.52	-
	8	1.57	0.61	-	-
	Mean(Y_i)	1.44	0.71	0.52	-
	S^2_i	0.064	0.021	0.147	-
	i	1	2	3	4

[1] Since the denominator of the proportion of females with eggs varies with the number of females occurring in that replicate, the adjustment of the arc sine square root transformation for 0% and 100% is not used for this data.

32. Replace Subsection 14.14.1.1.1 on page 294 of the marine chronic method manual with the following.

14.14.1.1.1 Data on the single-laboratory precision of the mysid survival, growth, and fecundity using copper (Cu) sulfate and sodium dodecyl sulfate (SDS) in natural seawater and in artificial seawater (GP2) are shown in Tables 29-33. ~~Survival NOEC/LOEC pairs showed good precision, and were the same in four of the six tests with Cu and SDS. Growth and fecundity were generally not acceptable endpoints in either sets of tests.~~ In Tables 29-30 the coefficient of variation for the IC25, ranges from 18.0 to 35.0 and the IC50, ranges from 5.8 to 47.8, indicating acceptable test precision. Data in Tables 31-33 show no detectable differences between tests conducted in natural or artificial seawaters.

33. Replace Subsection 15.5.2 on page 301 of the marine chronic method manual with the following.

15.5.2 Laboratory sea urchins, *Arbacia punctulata*, culture unit -- See Subsection ~~6.17~~ 15.6.19, culturing methods below and Section 4, Quality Assurance. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.

34. Replace Subsections 15.6.16.3.8 to 15.6.17.7 on page 308 of the marine chronic method manual with the following.

15.6.~~16~~ 18.3.8 Table 1 illustrates the preparation of test solutions at 30‰ if they are made by combining effluent (0‰), deionized water and HSB (100‰), or FORTY FATHOMS® sea salts.

15.6.~~16~~ 18.4 Artificial sea salts: FORTY FATHOMS® brand sea salts (Marine Enterprises, Inc., 8755 Mylander Lane, Baltimore, MD 21204; 301-321-1189) have been used successfully at the EMSL-Cincinnati, for long-term (6-12 months) maintenance of stock cultures of sexually mature sea urchins and to perform the sea urchin fertilization test. GP2 seawater formulation (Table 2) has also been used successfully at ERL-Narragansett, RI.

15.6.~~16~~ 18.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container -- not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26˚C. Seawater made from artifical sea salts is conditioned (Spotte, 1973; Spotte, et al., 1984; Bower, 1983).

15.6.~~16~~ 18.4.2 The GP2 reagent grade chemicals (Table ~~1~~2) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be between 21-26˚C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artifical seawater.

15.6.~~17~~ 19 TEST ORGANISMS, SEA URCHINS, *ARBACIA PUNCTULATA*

15.6.~~17~~ 19.1 Adult sea urchins, *Arbacia punctulata*, can be obtained from commercial suppliers. After acquisition, the animals are sexed by briefly stimulating them with current from a 12 V transformer.

Electrical stimulation causes the immediate release of masses of gametes that are readily identifiable by color -- the eggs are red, and the sperm are white.

15.6.~~17~~ 19.2 The sexes are separated and maintained in 20-L, aerated fiberglass tanks, each holding about 20 adults. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.~~17~~ 19.3 The culture unit should be maintained at 15 ± 3˚C, with a water temperature control device.

15.6.~~17~~ 19.4 The food consists of kelp, *Laminaria* sp., gathered from known uncontaminated zones or obtained from commercial supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at approximately one week intervals. Decaying food is removed as necessary. Ample supplies of food should always be available to the sea urchins.

15.6.~~17~~ 19.5 Natural or artificial seawater with a salinity of 30‰ is used to maintain the adult animals, for all washing and dilution steps, and as the control water in the tests (see Subsection 15.6.~~16~~ 18).

15.6.~~17~~ 19.6 Adult male and female animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to 15˚C before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.~~17~~ 19.7 To successfully maintain about 25 adult animals for 7 days at a field site, a screen-partitioned, 40-L glass aquarium using aerated, recirculating, clean saline water (30‰) and a gravel bed filtration system, is housed within a water bath, such as FORTY FATHOMS® or equivalent (15˚C). The inner aquarium is used to avoid contact of animals and water bath with cooling coils.

35. Replace Table 3 (continued) on page 318 of the marine chronic method manual with the following.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST WITH EFFLUENT AND RECEIVING WATERS (CONTINUED)

12. ~~Effluent~~ Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water or minimum of 5 and a control
13. ~~Test d~~ Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5
14. Test duration:	1 h and 20 min
15. Endpoint:	Fertilization of sea urchin eggs

16.	Test acceptability criteria:	70% - 90% egg fertilization in controls
17.	Sampling requirements:	For on-site tests, ~~O~~one sample collected at test initiation, and ~~preferably~~ used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)
18.	Sample volume required:	1 L per test

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36. Replace Table 3 on page 358 of the marine chronic method manual with the following.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION TEST WITH EFFLUENTS AND RECEIVING WATERS

1.	Test type:	Static, non-renewal
2.	Salinity:	30‰ (± 2‰ of the selected test salinity)
3.	Temperature:	23 ± 1˚C
~~4.~~	~~Photoperiod:~~	~~16 h light, 8 h darkness~~
~~5.~~	~~Light intensity:~~	~~75 µE/m²/s (500 ft-c)~~
~~6~~ 4.	Light ~~source~~ quality:	Cool-white fluorescent lights
5.	Light intensity:	75 µE/m^2/s (500 ft-c)
6.	Photoperiod:	16 h light, 8 h darkness
7.	Test chamber size:	200 mL polystyrene cups, or 250 mL Erlenmeyer flasks
8.	Test solution volume:	100 mL (minimum)
9.	No. organisms per test chamber:	5 female branch tips and 1 male plant
10.	No. replicate per concentration:	4 (minimum of 3)
11.	No. organisms per	

concentrations:	24 (minimum of 18)
12. Dilution water:	30‰ salinity natural seawater, or a combination of 50% of 30‰ salinity natural seawater and 50% of 30‰ salinity GP2 artificial seawater (see Section 7, Dilution Water)
13. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water or minimum of 5 and a control
14. ~~Test d~~ Dilution factor:	Effluents: ≥0.5 Receiving waters: None or ≥0.5

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37. Replace item 18 in Table 3 (continued) on page 359 of the marine chronic method manual with the following.

18. Sampling requirements:	For on-site tests, ~~O~~one sample collected at test initiation, and ~~preferably~~ used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)

38. Insert the following references into the Cited References section on page 380 of the marine chronic method manual.

Casarett, L.J. and J. Doull. 1975. Toxicology: the basic science of poisons. Macmillan Publishing Co., New York.

Lussier, S.M., A. Kuhn, and R. Comeleo. 1999. An evaluation of the seven-day toxicity test with *Americamysis bahia* (formerly *Mysidopsis bahia*). Environ. Toxicol. Chem. 18(12): 2888-2893.

Mount, D.R. and D.I. Mount. 1992. A simple method of pH control for static and static renewal aquatic toxicity tests. Environ. Toxicol. Chem. 11: 609-614.

Price, W.W., R.W. Heard, and L. Stuck. 1994. Observations on the genus *Mysidopsis* Sars, 1864 with the designation of a new genus, *Americamysis*, and the descriptions of *Americamysis alleni* and *Americamysis stucki* (Peracarida: Mysidacea: Mysidae), from the Gulf of Mexico. Proc. Biol. Soc. Wash. 107: 680-698.

USEPA. 1996. Marine toxicity identification evaluation (TIE): Phase I guidance document. R.M. Burgess, K.T. Ho, G.E. Morrison, G. Chapman, and D.L. Denton (eds.). National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Narragansett, RI. EPA/600/R-96/054.

USEPA. 2000a. Method guidance and recommendations for whole effluent toxicity (WET) testing (40 CFR Part 136). Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-00/004.

USEPA. 2000b. Understanding and accounting for method variability in whole effluent toxicity applications under the national pollutant discharge elimination system program. Office of Wastewater Management, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/833/R-00/003.

USEPA. 2001a. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 1. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

USEPA. 2001b. Final report: interlaboratory variability study of EPA short-term chronic and acute whole effluent toxicity test methods, Vol. 2: Appendix. Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 20460. EPA/821/B-01/004.

III. Precision

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Replace Subsection 4.13.4 on page 12 of the acute method manual with the following.

4.13.4 Inter-laboratory precision of acute toxicity tests from 253 reference toxicant tests with seven species, listed in Tables 2, 3, 4, and 5 (expressed as CV% for LC50s), ranged from 11% to 167%. Table 6 shows interlaboratory precision data from a study of acute toxicity test methods using reference toxicant, effluent, and receiving water sample types (USEPA, 2001a; USEPA, 2001b). Averaged across sample types, total interlaboratory precision (expressed as CV% for LC50s) ranged from 13% to 38.5% for the acute methods.

2. Insert the following table after Table 5 on page 17 of the acute method manual.

TABLE 6. NATIONAL INTERLABORATORY STUDY OF ACUTE TOXICITY TEST PRECISION, 2000: PRECISION OF LC50 POINT ESTIMATES FOR REFERENCE TOXICANT, EFFLUENT, AND RECEIVING WATER SAMPLE TYPES[1].

Method	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
Pimephales promelas	KCl	7.62	19.7	21.1
	Municipal effluent	10.3	19.2	21.8
	Receiving water	-	-	17.2
	Average	8.96	19.4	20.0
Ceriodaphnia dubia	KCl	14.6	15.2	21.1
	Municipal effluent	9.68	32.8	34.2
	Receiving water	-	-	31.8
	Average	12.1	24.0	29.0

Cyprinodon variegatus	KCl	-	-	26.0
	Municipal effluent	-	-	19.4
	Receiving water	-	-	32.5
	Average	-	-	26.0
Menidia beryllina	$CuSO_4$[6]	-	-	-
	Industrial effluent	9.91	49.7	50.7
	Receiving water	-	-	26.3
	Average	9.91	49.7	38.5
Holmesimysis costata[7]	Zn (48 h test)	19	-	
	Zn (96 h test)	23	-	
	Zn (interlaboratory trial 1)	-	-	24
	Zn (interlaboratory trial 2)	-	-	1
	Average	21		13

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type. The study design also did not provide within-laboratory replication for the *Cyprinodon variegatus* Acute Method.

[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

[6] Precision estimates were not calculated for the reference toxicant sample type since the majority of results for this sample type were outside of the test concentration range (ie., >100).

[7] *Holmesimysis costata* Acute Test data were from Martin *et al.* (1989). Zn was tested in two intralaboratory trials and in two interlaboratory trials. Data from this study was only reported to two significant figures.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Replace Subsection 4.14.3 on page 14 of the freshwater chronic method manual with the following.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests with two species using the reference toxicants potassium chloride and copper sulfate are shown in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). The effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

2. Insert the following table after Table 1 on page 15 of the freshwater chronic method manual.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1].

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Pimephales promelas	Growth, IC25	73	20.9
Ceriodaphnia dubia	Reproduction, IC25	34	35.0
Selenastrum capricornutum (with EDTA)	Growth, IC25	21	34.3
	Growth, IC50	22	32.2
Selenastrum capricornutum (without EDTA)	Growth, IC25	21	58.5
	Growth, IC50	22	58.5

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

[3] CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

3. Insert the following into Subsection 11.14.1, Precision, on page 108 of the freshwater chronic method manual.

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

4. Insert the following into Subsection 11.14.1.1, Single-Laboratory Precision, on page 108 of the freshwater chronic method manual.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 19 laboratories (USEPA, 2000b). The database consisted of 205 reference toxicant tests conducted in 19 laboratories using a variety of reference toxicants including: cadmium, chromium, copper, potassium chloride, sodium chloride, sodium pentachlorophenate, and sodium dodecyl sulfate. Among the 19 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 21%; and in 75% of laboratories, the within-laboratory CV was less than 38%.

5. Insert the following into Subsection 11.14.1.2, Multilaboratory Precision, on page 111 of the freshwater chronic method manual.

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 27 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 101 Fathead Minnow Larval Survival and Growth tests conducted in this study, 98.0% were successfully completed and met the required test acceptability criteria. Of 24 tests that were conducted on blank samples, none showed false

positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 4.35%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 22 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 20.9% for IC25 results. Table 23 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 97.2%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type and four concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 86.1%, 91.7%, and 76.9% for the reference toxicant, effluent, and receiving water sample types, respectively.

6. Replace the footnotes to Table 20 on page 112 of the freshwater chronic method manual with the following.

1 From DeGraeve et al., 1988.

~~2 Percent of values within one concentration intervals of the median.~~

~~3 Percent of values within two or more concentrations intervals of the median.~~

2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

3 Percent of values two or more concentration intervals above or below the median.

7. Replace the footnotes to Table 21 on page 113 of the freshwater chronic method manual with the following.

1 From DeGraeve et al., 1988.

~~2 Percent of values within one concentration intervals of the median.~~

~~3 Percent of values within two or more concentrations intervals of the median.~~

2 Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

3 Percent of values two or more concentration intervals above or below the median.

8. Insert the following tables after Table 21 on page 113 of the freshwater chronic method manual.

TABLE 22. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.0	17.2	19.9

Effluent		19.1	12.9	23.1
Receiving water		-	-	19.8
	Average	14.6	15.0	20.9

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

[3] The within-laboratory component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 23. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	50%	75.0	22.2	2.78
	Effluent	12.5%	76.9	23.1	0.00
	Receiving water	25%	69.2	30.8	0.00
Growth NOEC	Reference toxicant	50%	58.3	27.8	13.9
	Effluent	12.5%	66.7	25.0	8.33
	Receiving water	12.5%	30.8	46.1	23.1

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

9. Insert the following into Subsection 13.14.1, Precision, on page 189 of the freshwater chronic method manual.

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

10. Insert the following into Subsection 13.14.1.1, Single-Laboratory Precision, on page 189 of the freshwater chronic method manual.

13.14.1.1.3 EPA evaluated within-laboratory precision of the Daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test using a database of routine reference toxicant test results from 33 laboratories (USEPA, 2000b). The database consisted of 393 reference toxicant tests conducted in 33 laboratories using a variety of reference toxicants including: cadmium, copper, potassium chloride, sodium chloride, and sodium pentachlorophenate. Among the 33 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 reproduction endpoint. In 25% of laboratories, the within-laboratory CV was less than 17%; and in 75% of laboratories, the within-laboratory CV was less than 45%.

11. Insert the following into Subsection 13.14.1.2, Multilaboratory Precision, on page 192 of the freshwater chronic method manual.

13.14.1.2.3 In 2000, EPA conducted an interlaboratory variability study of the Daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 34 participant laboratories tested 3 or 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Of the 122 *Ceriodaphnia dubia* Survival and Reproduction tests conducted in this study, 82.0% were successfully completed and met the required test acceptability criteria. Of 27 tests that were conducted on blank samples, none showed false positive results for survival endpoints, and only one resulted in false positive results for the growth endpoint, yielding a false positive rate of 3.70%. Results from the reference toxicant, effluent, and

receiving water sample types were used to calculate the precision of the method. Table 20 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 35.0% for IC25 results. Table 21 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant and effluent sample types and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 97.2%, 91.3%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned five concentrations for the reference toxicant sample type, three concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 83.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

12. Insert the following tables after Table 19 on page 195 of the freshwater chronic method manual.

TABLE 20. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	-	-	-
	Effluent	17.4	27.6	32.6
	Receiving water	-	-	37.4
	Average	17.4	27.6	35.0

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the reference toxicant sample type a majority of the results were outside of the test concentration range, so precision estimates were not calculated. For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 21. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	100%	97.2	0.00	2.78
	Effluent	25%	65.2	26.1	8.70
	Receiving water	25%	90.0	10.0	0.00
Growth NOEC	Reference toxicant	100%	72.2	11.1	16.7
	Effluent	12.5%	70.8	29.2	0.00
	Receiving water	25%	70.0	30.0	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

13. Insert the following into Subsection 14.14.1, Precision, on page 225 of the freshwater chronic method manual.

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

14. Insert the following into Subsection 14.14.1.1, Single-Laboratory Precision, on page 225 of the freshwater chronic method manual.

14.14.1.1.2 EPA evaluated within-laboratory precision of the green alga, *Selenastrum capricornutum*, Growth Test using a database of routine reference toxicant test results from nine laboratories (USEPA, 2000b). The database consisted of 85 reference toxicant tests conducted in 9 laboratories using a variety of reference toxicants including: copper, sodium chloride, and zinc. Among the 9 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 26% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 25%; and in 75% of laboratories, the within-laboratory CV was less than 39%.

15. Replace Subsection 14.14.1.2.1 on page 225 of the freshwater chronic method manual with the following.

14.14.1.2.1 ~~Data on the multilaboratory precision of this test are not yet available.~~ In 2000, EPA conducted an interlaboratory variability study of the green alga, *Selenastrum capricornutum*, Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of moderately-hard synthetic freshwater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample consisted of moderately-hard synthetic freshwater spiked with KCl. Each sample was tested with and without the addition of EDTA. Of the 44 *Selenastrum capricornutum* Growth tests conducted with EDTA, 63.6% were successfully completed and met the required test acceptability criteria. Of the 44 tests conducted without EDTA, 65.9% were successfully completed and met the required test acceptability criteria. Of five tests that were conducted on blank samples with the addition of EDTA, none showed false positive results for the growth endpoint. Of 6 tests that were conducted on blank samples without the addition of EDTA, 2 showed false positive results for the growth endpoint, yielding a false positive rate of 33.3%. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 13 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 34.3% and 58.5% for IC25 results in tests with EDTA and without EDTA, respectively. Table 14 shows the precision of growth NOEC endpoints for each sample type. NOEC values for tests with EDTA spanned three concentrations for the effluent sample type and four concentrations for the reference toxicant and receiving water sample types. NOEC values for tests without EDTA, spanned six concentrations for the reference toxicant sample type, four concentrations for the effluent sample type, and two concentrations for the receiving water sample type. The percentage of values within one concentration of the median for tests conducted with EDTA was 85.7%, 100%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. The percentage of values within one concentration of the median for tests conducted without EDTA was 40.0%, 50.0%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively.

16. Insert the following tables after Table 12 on page 228 of the freshwater chronic method manual.

TABLE 13. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	10.9	20.8	23.5
(with EDTA)	Effluent	39.5	8.48	40.4
	Receiving water	-	-	38.9
	Average	25.2	14.6	34.3
IC25	Reference toxicant	25.6	83.6	87.5
(without EDTA)	Effluent	21.0	60.3	63.9
	Receiving water	-	-	24.1
	Average	23.3	72.0	58.5

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 14. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Growth NOEC (with EDTA)	Reference toxicant	25%	57.1	28.6	14.3
	Effluent	6.25%	42.9	57.1	0.00
	Receiving water	12.5%	28.6	57.1	14.3
Growth NOEC (without EDTA)	Reference toxicant	18.8%	-[4]	40.0	60.0
	Effluent	18.8%	-[4]	50.0	50.0
	Receiving water	6.25%	75.0	25.0	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.
[4] The median NOEC fell between test concentrations, so no test results fell precisely on the median.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Replace Subsection 4.14.3 on page 17 of the marine chronic method manual with the following.

4.14.3 Interlaboratory precision data from a 1991 study of chronic toxicity tests using two reference toxicants with the mysid, *Mysidopsis bahia*, and the inland silverside, *Menidia beryllina*, is listed in Table 1. Table 2 shows interlaboratory precision data from a study of three chronic toxicity test methods using effluent, receiving water, and reference toxicant sample types (USEPA, 2001a; USEPA, 2001b). For the *Mysidopsis bahia* and the *Cyprinodon variegatus* test methods, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a river water spiked with KCl, and the reference toxicant sample was bioassay-grade Forty Fathoms® synthetic seawater spiked with KCl. For the *Menidia beryllina* test method, the effluent sample was an industrial wastewater spiked with $CuSO_4$, the receiving water sample was a natural seawater spiked with $CuSO_4$, and the reference toxicant sample was bioassay-grade Forty Fathoms® synthetic seawater spiked with $CuSO_4$. Additional precision data for each of the tests described in this manual are presented in the sections describing the individual test methods.

2. Insert the following table after Table 1 on page 18 of the marine chronic method manual.

TABLE 2. NATIONAL INTERLABORATORY STUDY OF CHRONIC TOXICITY TEST PRECISION, 2000: PRECISION OF RESPONSES USING EFFLUENT, RECEIVING WATER, AND REFERENCE TOXICANT SAMPLE TYPES[1].

Organism	Endpoint	Number of Tests[2]	CV (%)[3]
Cyprinodon variegatus	Growth, IC25	21	10.5
Menidia beryllina	Growth, IC25	30	43.8
Mysidopsis bahia	Growth, IC25	36	41.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Represents the number of valid tests (i.e., those that met test acceptability criteria) that were used in the analysis of precision. Invalid tests were not used.

[3] CVs based on total interlaboratory variability (including both within-laboratory and between-laboratory components of variability) and averaged across sample types. IC25s or IC50s were pooled for all laboratories to calculate the CV for each sample type. The resulting CVs were then averaged across sample types.

3. Insert the following into Subsection 11.14.1, Precision, on page 116 of the marine chronic method manual.

11.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 11.14.1.1 and 11.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

4. Insert the following into Subsection 11.14.1.1, Single-Laboratory Precision, on page 116 of the marine chronic method manual.

11.14.1.1.2 EPA evaluated within-laboratory precision of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test using a database of routine reference toxicant test results

from five laboratories (USEPA, 2000b). The database consisted of 57 reference toxicant tests conducted in 5 laboratories using reference toxicants including: cadmium and potassium chloride. Among the 5 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 13% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 9%; and in 75% of laboratories, the within-laboratory CV was less than 14%.

5. Insert the following into Subsection 11.14.1.2, Multilaboratory Precision, on page 116 of the marine chronic method manual.

11.14.1.2.2 In 2000, EPA conducted an interlaboratory variability study of the Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 7 participant laboratories tested 4 blind test samples that included blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade Forty Fathoms® synthetic seawater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a natural seawater spiked with KCl, and the reference toxicant sample consisted of bioassay-grade Forty Fathoms® synthetic seawater spiked with KCl. Of the 28 Sheepshead Minnow Larval Survival and Growth tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of 7 tests that were conducted on blank samples, none showed false positive results for the survival endpoint or the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 28 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 10.5% for IC25 results. Table 29 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned two concentrations for the reference toxicant sample type and one concentration for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types. For the growth endpoint, NOEC values spanned one concentration for the reference toxicant sample type and two concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types.

6. Insert the following tables after Table 27 on page 123 of the marine chronic method manual.

TABLE 28. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]
IC25	Reference toxicant	18.4
	Effluent	6.12
	Receiving water	7.15
	Average	10.5

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the total interlaboratory variability (including both within-laboratory and between-laboratory components of variability). Individual within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

TABLE 29. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	25%	57.1	42.9	0.00
	Effluent	25%	100	0.00	0.00
	Receiving water	25%	100	0.00	0.00
Growth NOEC	Reference toxicant	25%	100	0.00	0.00
	Effluent	12.5%	57.1	42.9	0.00
	Receiving water	12.5%	71.4	28.6	0.00

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).
[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.
[3] Percent of values two or more concentration intervals above or below the median.

7. Insert the following into Subsection 13.14.1, Precision, on page 216 of the marine chronic method manual.

13.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 13.14.1.1 and 13.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these

studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

8. Insert the following into Subsection 13.14.1.1, Single-Laboratory Precision, on page 216 of the marine chronic method manual.

13.14.1.1.2 EPA evaluated within-laboratory precision of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test using a database of routine reference toxicant test results from 16 laboratories (USEPA, 2000b). The database consisted of 193 reference toxicant tests conducted in 16 laboratories using a variety of reference toxicants including: chromium, copper, potassium chloride, and sodium dodecyl sulfate. Among the 16 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 27% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 18%; and in 75% of laboratories, the within-laboratory CV was less than 43%.

9. Replace Subsection 13.14.1.2.1 on page 216 of the marine chronic method manual with the following.

13.14.1.2.1 ~~Data on the multilaboratory precision of the inland silverside larval survival and growth test are not yet available.~~ In 2000, EPA conducted an interlaboratory variability study of the Inland Silverside, *Menidia beryllina*, Larval Survival and Growth Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 10 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade Forty Fathoms® synthetic seawater, the effluent sample was an industrial wastewater spiked with $CuSO_4$, the receiving water sample was a natural seawater spiked with $CuSO_4$, and the reference toxicant sample consisted of bioassay-grade Forty Fathoms® synthetic seawater spiked with $CuSO_4$. Of the 40 *Menidia beryllina* Larval Survival and Growth tests conducted in this study, 100% were successfully completed and met the required test acceptability criteria. Of seven tests that were conducted on blank samples, none showed false positive results for survival endpoints or for the growth endpoint. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 23 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 43.8% for IC25 results. Table 24 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned five concentrations for the effluent, four concentrations for the reference toxicant sample type, and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 84.6%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively. For the growth endpoint, NOEC values spanned four concentrations for the reference toxicant and effluent sample types and three concentrations for the receiving water sample type. The percentage of values within one concentration of the median was 90.9%, 91.7%, and 85.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

10. Insert the following tables after Table 22 on page 221 of the marine chronic method manual.

TABLE 23. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25	Reference toxicant	22.0	29.1	36.4
	Effluent	7.24	55.5	56.0
	Receiving water	-	-	39.1
	Average	14.6	42.3	43.8

1 From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

2 CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

3 The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

4 The between-laboratory component of variability for duplicate samples tested at different laboratories.

5 The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 24. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	12.5%	72.7	18.2	9.09
	Effluent	25%	38.5	46.1	15.4
	Receiving water	25%	57.1	28.6	14.3

Growth NOEC	Reference toxicant	12.5%	72.7	18.2	9.09
	Effluent	25%	41.7	50.0	8.33
	Receiving water	25%	57.1	28.6	14.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

11. Insert the following into Subsection 14.14.1, Precision, on page 294 of the marine chronic method manual.

14.14.1 PRECISION – Data on single-laboratory and multilaboratory precision are described below (Subsections 14.14.1.1 and 14.14.1.2). Single-laboratory precision is a measure of the reproducibility of test results when tests are conducted using a specific method under reasonably constant conditions in the same laboratory. Single-laboratory precision is synonymous with the terms within-laboratory precision and intralaboratory precision. Multilaboratory precision is a measure of the reproducibility of test results from different laboratories using the same test method and analyzing the same test material. Multilaboratory precision is synonymous with the term interlaboratory precision. Interlaboratory precision, as used in this document, includes both within-laboratory and between-laboratory components of variability. In recent multilaboratory studies, these two components of interlaboratory precision have been displayed separately (termed within-laboratory and between-laboratory variability) and combined (termed total interlaboratory variability). The total interlaboratory variability that is reported from these studies is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

12. Insert the following into Subsection 14.14.1.1, Single-Laboratory Precision, on page 294 of the marine chronic method manual.

14.14.1.1.2 EPA evaluated within-laboratory precision of the Mysid, *Mysidopsis bahia*, Survival, Growth, and Fecundity Test using a database of routine reference toxicant test results from 10 laboratories (USEPA, 2000b). The database consisted of 130 reference toxicant tests conducted in 10 laboratories using a variety of reference toxicants including: chromium, copper, and potassium chloride. Among the 10 laboratories, the median within-laboratory CV calculated for routine reference toxicant tests was 28% for the IC25 growth endpoint. In 25% of laboratories, the within-laboratory CV was less than 24%; and in 75% of laboratories, the within-laboratory CV was less than 32%.

13. Replace Subsection 14.14.1.2.1 on page 294 of the marine chronic method manual with the following.

14.14.1.2.1 ~~The multilaboratory precision of the test has not yet been determined.~~ In 2000, EPA conducted an interlaboratory variability study of the Mysid, *Mysidopsis bahia*, Survival, Growth, and

Fecundity Test (USEPA, 2001a; USEPA, 2001b). In this study, each of 11 participant laboratories tested 4 blind test samples that included some combination of blank, effluent, reference toxicant, and receiving water sample types. The blank sample consisted of bioassay-grade Forty Fathoms® synthetic seawater, the effluent sample was a municipal wastewater spiked with KCl, the receiving water sample was a natural seawater spiked with KCl, and the reference toxicant sample consisted of bioassay-grade Forty Fathoms® synthetic seawater spiked with KCl. Of the 44 *Mysidopsis bahia* Survival, Growth, and Fecundity tests conducted in this study, 97.7% were successfully completed and met the required test acceptability criteria. Of seven tests that were conducted on blank samples, none showed false positive results for survival, growth, or fecundity endpoints. Results from the reference toxicant, effluent, and receiving water sample types were used to calculate the precision of the method. Table 34 shows the precision of the IC25 for each of these sample types. Averaged across sample types, the total interlaboratory variability (expressed as a CV%) was 41.3% for growth IC25 results. Table 35 shows the frequency distribution of survival and growth NOEC endpoints for each sample type. For the survival endpoint, NOEC values spanned three concentrations for the reference toxicant, effluent, and receiving water sample types. The percentage of values within one concentration of the median was 100% for each of the sample types. For the growth endpoint, NOEC values spanned four concentrations for the reference toxicant sample type and three concentrations for the effluent and receiving water sample types. The percentage of values within one concentration of the median was 92.3%, 100%, and 100% for the reference toxicant, effluent, and receiving water sample types, respectively. For the fecundity endpoint, NOEC values spanned three concentrations for the reference toxicant, the effluent, and the receiving water sample types. The percentage of values within one concentration of the median was 75.0%, 87.5%, and 66.7% for the reference toxicant, effluent, and receiving water sample types, respectively.

14. Insert the following tables after Table 33 on page 299 of the marine chronic method manual.

TABLE 34. PRECISION OF POINT ESTIMATES FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	CV (%)[2]		
		Within-lab[3]	Between-lab[4]	Total[5]
IC25 for Growth	Reference toxicant	8.69	40.0	40.9
	Effluent	5.26	36.6	37.0
	Receiving water	-	-	45.9
	Average	6.98	38.3	41.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] CVs were calculated based on the within-laboratory component of variability, the between-laboratory component of variability, and the total interlaboratory variability (including both within-laboratory and between-laboratory components). For the receiving water sample type, within-laboratory and between-laboratory components of variability could not be calculated since the study design did not provide within-laboratory replication for this sample type.

[3] The within-laboratory (intralaboratory) component of variability for duplicate samples tested at the same time in the same laboratory.

[4] The between-laboratory component of variability for duplicate samples tested at different laboratories.

[5] The total interlaboratory variability, including within-laboratory and between-laboratory components of variability. The total interlaboratory variability is synonymous with interlaboratory variability reported from other studies where individual variability components are not separated.

TABLE 35. FREQUENCY DISTRIBUTION OF HYPOTHESIS TESTING RESULTS FOR VARIOUS SAMPLE TYPES[1].

Test Endpoint	Sample Type	Median NOEC Value	% of Results at the Median	% of Results ±1[2]	% of Results ≥2[3]
Survival NOEC	Reference toxicant	25%	53.8	46.2	0.00
	Effluent	12.5%	46.7	53.3	0.00
	Receiving water	12.5%	37.5	62.5	0.00
Growth NOEC	Reference toxicant	25%	53.8	38.5	7.69
	Effluent	12.5%	46.7	53.3	0.00
	Receiving water	12.5%	50.0	50.0	0.00
Fecundity NOEC	Reference toxicant	18.8%	-[4]	75.0	25.0
	Effluent	25%	62.5	25.0	12.5
	Receiving water	9.38%	-[4]	66.7	33.3

[1] From EPA's WET Interlaboratory Variability Study (USEPA, 2001a; USEPA, 2001b).

[2] Percent of values at one concentration interval above or below the median. Adding this percentage to the percent of values at the median yields the percent of values within one concentration interval of the median.

[3] Percent of values two or more concentration intervals above or below the median.

[4] The median NOEC fell between test concentrations, so no test results fell precisely on the median.

15. Insert the following into Subsection 16.14.1.1, Single-Laboratory Precision, on page 373 of the marine chronic method manual.

16.14.1.1.2 EPA evaluated single-laboratory (within-laboratory) precision of the Red Macroalga, *Champia parvula*, Reproduction Test using a database of routine reference toxicant test results from two laboratories (USEPA, 2000b). The database consisted of 23 reference toxicant tests conducted in 2 laboratories using reference toxicants including: copper and sodium dodecyl sulfate. The within-laboratory CVs calculated for routine reference toxicant tests at these 2 laboratories were 58% and 59% for the IC25 reproduction endpoint.

IV. Blocking by Known Parentage

A. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Replace Figure 1 on page 156 of the freshwater chronic method manual with the following.

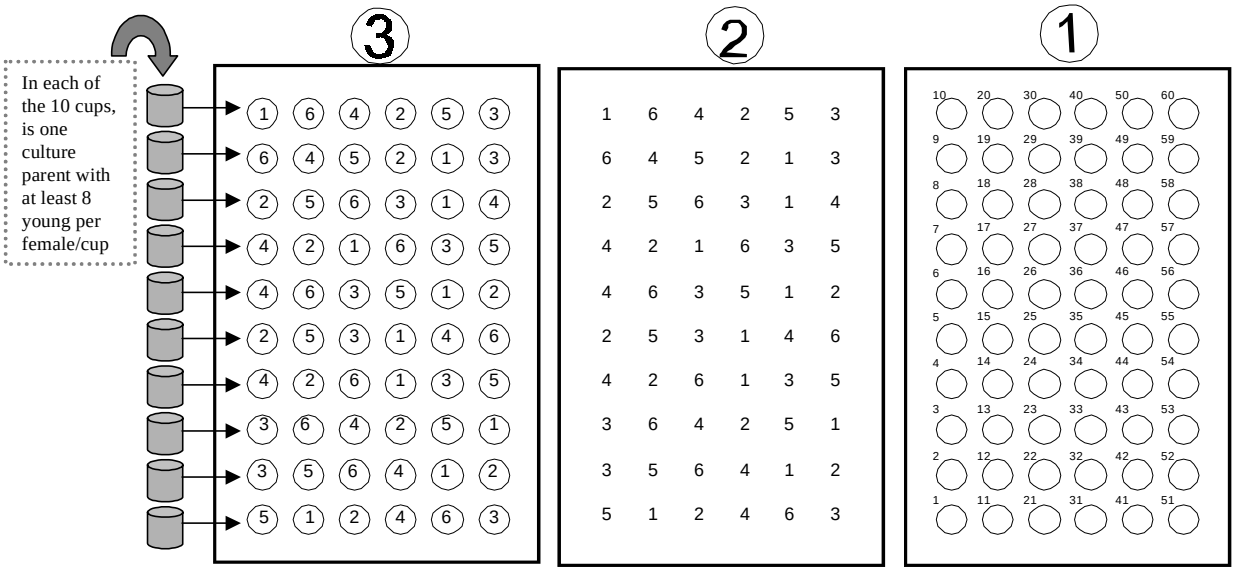

Figure 1. Examples of a test board and randomizing template: ~~(1)~~ 1) test board with positions for six columns of ten replicate test chambers with each position numbered for recording results on data sheets, ~~(2)~~ 2) cardboard randomizing template prepared by ~~throwing a single die~~ randomly drawing numbers (1-6) for each position in ~~each~~ a row across the board, and ~~(3)~~ 3) test board (1) placed on top of the randomizing template (2) for the purpose of assigning the position of ~~treatment solutions (cups)~~ test treatments (1-6) within each ~~row on the board~~ block (row on the test board). Following placement of test chambers, test organisms are allocated using blocking by known parentage. Test organisms from a single brood cup are distributed to each treatment within a given block (row on the test board).

2. Replace Subsection 13.10.2, Start of the Test, on page 160 of the freshwater chronic method manual with the following.

13.10.2 START OF THE TEST

13.10.2.1 Label the test chambers with a marking pen. Use of color-coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) must have ten replicates.

13.10.2.2 The test ~~solutions~~ chambers ~~can~~ must be randomly assigned to a board using a template (Figure 1) or by using ~~a table of~~ random numbers (see Appendix A). ~~When using the randomized block design, test chambers are randomized only once, at the beginning of the test.~~ Randomizing the position of test chambers as described in Figure 1 (or equivalent) will assist in assigning test organisms using blocking by known parentage (Subsection 13.10.2.4). A number of different templates should be prepared, ~~so that the same template is not used for every test.~~ and the template used for each test should be identified on the data sheet. The same template must not be used for every test.

13.10.2.3 Neonates less than 24 h old, and all within 8 h of the same age, are required to begin the test. The neonates ~~are~~ must be obtained from individual cultures using brood boards, as described above in Subsection 13.6.16.6, Individual Culture (also see Section 6, Test Organisms). Neonates ~~are~~ must be taken only from adults in individual cultures that have eight or more young in their third or subsequent broods. These adults can be used as brood stock until they are 14 days old. If the neonates are held more than one or two hours before using in the test, they should be fed (0.1 mL YCT and 0.1 mL algal concentrate/15 mL of media). Record the age of test organisms, source, and feeding of neonates on test data sheets.

13.10.2.4 Ten brood cups, each with 8 or more young, are randomly selected from a brood board for use in setting up a test. To start the test, neonates from these ten brood cups are distributed to each test chamber on the test board (one per test chamber). Test organisms must be assigned to test chambers using a block randomization procedure, such that offspring from a single female are distributed evenly among the treatments, appearing once in every test concentration. This arrangement is referred to as "blocking by known parentage". The technique used to achieve blocking by known parentage should be recorded in the test data report. One effective technique is to block randomize the test board as described in Figure 1 and transfer one neonate from the first brood cup ~~is transferred~~ to each of the six test chambers in the first row on the test board ~~(Figure 1)~~. One neonate from the second brood cup is then transferred to each of the six test chambers in the second row on the test board. This process is continued until each of the 60 test chambers contains one neonate. The set of six test chambers (one for each test treatment) containing organisms derived from a single female parent is referred to as a block. When using the technique described in Figure 1, each row of the test board will represent a block.

13.10.2.4.1 The brood cups and test chambers may be placed on a light table to facilitate counting the neonates. However, care must be taken to avoid temperature increase due to heat from the light table.

13.10.2.4.2 Following the allocation of test organisms to the test board, additional neonates might remain in the ten brood cups that were selected for test setup. These additional neonates may be discarded or used as future culture organisms if needed.

13.10.2.5 ~~This blocking procedure~~ Blocking by known parentage allows the performance of each ~~female~~ test organism to be tracked to its parent culture organism. This technique ensures that any brood effects (i.e., differences in test organism fecundity or sensitivity attributable to the source of parentage) are evenly distributed among the test treatments. ~~If a female produces one weak offspring or male, the likelihood of producing all weak offspring or all males is greater. By using this known parentage technique~~ Also, by knowing the parentage of each test organism, ~~poor performance of young from a given female~~ blocks consisting largely of males can be omitted from all ~~concentrations.~~ test treatments at the end of the test (see Subsection 13.13.1.4), decreasing variability among replicates.

3. Insert the following into Subsection 13.10.9, Termination of the Test, on page 165 of the freshwater chronic method manual.

13.10.9.3.1 In general, the occurrence of males in healthy, well-maintained individual cultures is rare. In interlaboratory testing of the *Ceriodaphnia dubia* Survival and Reproduction Test, males were identified in only 7% (9 of 126 tests) of tests conducted (USEPA, 2001a). The number of males identified in these tests ranged from 1 to 12. In five tests containing a large number of males (4-12), laboratories conducting those tests also noted that organism cultures were experiencing or recovering from some stress. Since male production in cladoceran populations is generally associated with conditions of environmental stress (Pennak, 1989), culture conditions should be examined whenever males are identified in a test.

4. Replace item 10 in Table 3 on page 168 of the freshwater chronic method manual with the following.

10. No. neonates per test chamber	1. Assigned using blocking by known parentage (Subsection 13.10.2.4).

5. Insert the following into Subsection 13.13.1, General, on page 170 of the freshwater chronic method manual.

13.13.1.4 At the end of the test, if 50% or more of the surviving organisms in a block are identified as males, the entire block must be excluded from data analysis for the reproduction endpoint (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of the survival endpoint (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). For blocks having fewer than 50% of surviving organisms identified as males, the males (not the entire block) must be excluded from the analysis of reproduction (i.e., calculation of the reproduction NOEC and IC25 as described in Subsection 13.13.3), but may be used in the analysis of survival (i.e., calculation of the survival NOEC and LC50 as described in Subsection 13.13.2). Note that the exclusion of males from the analysis of reproduction may create unequal sample sizes among the concentrations, influencing the statistical methods chosen for analysis of reproduction (Figure 6). Determinations regarding test acceptability criteria for survival and reproduction (Subsection 13.12) must be made prior to exclusion of any blocks. In addition to these test acceptability criteria, if fewer than eight replicates in the control remain after excluding males and blocks with 50% or more of surviving organisms identified as males, the test is invalid and must be repeated with a newly collected sample.

V. pH Drift

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Replace Subsection 9.5.9 on page 49 of the acute method manual with the following.

9.5.9 pH drift ~~increases in pH may occur in test solutions~~ during acute, static-renewal, or non-renewal toxicity tests may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present ~~of pollutants such as ammonia~~. This problem can be minimized by conducting a test in a static-renewal mode rather than a non-renewal mode, or the problem can be avoided by conducting a test in a ~~static renewal or~~ flow through mode rather than a static-renewal or non-renewal mode.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 11.3, Interferences, on page 58 of the freshwater chronic method manual.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after warming to test temperature.

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was

effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

11.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

11.3.6.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). pH can be controlled using the CO_2-controlled atmosphere technique by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992). Prior experimentation will be needed to determine the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, CO_2 is injected to maintain the test pH at the initial pH of the sample after the sample has warmed to test temperature. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

11.3.6.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2.

2. Insert the following into Subsection 12.3, Interferences, on page 114 of the freshwater chronic method manual.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also

increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after warming to test temperature.

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

12.3.5.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). pH can be controlled using the CO_2-controlled atmosphere technique by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992). Prior experimentation will be needed to determine the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine

the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, CO_2 is injected to maintain the test pH at the initial pH of the sample after the sample has warmed to test temperature. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.3.5.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2.

3. Insert the following into Subsection 13.3, Interferences, on page 144 of the freshwater chronic method manual.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after warming to test temperature.

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.2 pH units in pH-controlled tests (USEPA, 1992).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

13.3.6.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). pH can be controlled using the CO_2-controlled atmosphere technique by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of CO_2 into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992). Prior experimentation will be needed to determine the appropriate volume of CO_2 to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents. If more than 5% CO_2 is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% CO_2 (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, CO_2 is injected to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, CO_2 is injected to maintain the test pH at the initial pH of the sample after the sample has warmed to test temperature. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.3.6.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 11.3, Interferences, on page 61 of the marine chronic method manual.

11.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at

pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

11.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 11.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after adjusting the sample salinity for use in marine testing.

11.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.3 pH units in pH-controlled tests (USEPA, 1996).

11.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

11.3.6.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 11.3.6.2) is applied routinely to subsequent testing of the effluent.

11.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). pH can be controlled using the CO_2-controlled atmosphere technique by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio to control pH at the target level. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the initial pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and

Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

11.3.6.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 11.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 11.3.6.2.

2. Insert the following into Subsection 12.3, Interferences, on page 124 of the marine chronic method manual.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after adjusting the sample salinity for use in marine testing.

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.3 pH units in pH-controlled tests (USEPA, 1996).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

12.3.5.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). pH can be controlled using the CO_2-controlled atmosphere technique by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio to control pH at the target level. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the initial pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

12.3.5.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2.

3. Insert the following into Subsection 13.3, Interferences, on page 163 of the marine chronic method manual.

13.3.6 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

13.3.6.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 13.3.6.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after adjusting the sample salinity for use in marine testing.

13.3.6.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.3 pH units in pH-controlled tests (USEPA, 1996).

13.3.6.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

13.3.6.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 13.3.6.2) is applied routinely to subsequent testing of the effluent.

13.3.6.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). pH can be controlled using the CO_2-controlled atmosphere technique by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio to control pH at the target level. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the initial pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

13.3.6.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 13.3.6.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 13.3.6.2.

4. Insert the following into Subsection 14.3, Interferences, on page 222 of the marine chronic method manual.

14.3.4 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 14.3.4.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 14.3.4.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

14.3.4.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 14.3.4.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, the pH should be maintained at the initial pH of the sample after adjusting the sample salinity for use in marine testing.

14.3.4.1.1 During parallel testing, the pH must be measured in each treatment at the beginning and end of each 24-h exposure period. pH measurements taken during the test must confirm that (1) pH was effectively maintained at the target pH in the controlled-pH treatment, and (2) pH drift in the uncontrolled-pH treatment was substantially greater than in the controlled-pH treatment. In general, the range in pH (i.e., drift) occurring in the uncontrolled-pH treatment must be at least twice that of the range observed in the controlled-pH treatment. Test procedures for conducting toxicity identification evaluations (TIEs) recommend maintaining pH within ±0.3 pH units in pH-controlled tests (USEPA, 1996).

14.3.4.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent may indicate ammonia toxicity (USEPA, 1992).

14.3.4.1.3 Following parallel testing, the toxicity observed in the controlled and uncontrolled-pH treatments is compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information

or additional parallel testing before pH control (as described in Subsection 14.3.4.2) is applied routinely to subsequent testing of the effluent.

14.3.4.2 The pH can be controlled with the addition of acids and bases and/or the use of a CO_2-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.9). pH can be controlled using the CO_2-controlled atmosphere technique by placing test chambers in an atmosphere flushed with a predetermined mixture of CO_2 and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate CO_2/air ratio to control pH at the target level. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the pH of the receiving water. If the objective of the WET test is to determine the absolute (end-of-pipe) toxicity of the effluent, atmospheric CO_2 in the test chambers is adjusted to maintain the test pH at the initial pH of the sample after adjusting the sample salinity for use in marine testing. USEPA (1996) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a CO_2-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

14.3.4.3 In rare circumstances, the daily cycle of upward pH drift and renewal (which returns the test pH to the initial sample pH) may cause artifactual toxicity even in the absence of pH-dependent toxicants. If toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 14.3.4.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 14.3.4.2.

VI. Concentration-response Relationships

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Replace Section 12, Report Preparation, on page 119 of the acute method manual with the following.

SECTION 12
REPORT PREPARATION AND TEST REVIEW

12.1 REPORT PREPARATION

The following general format and content are recommended for the report:

12.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address

12.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

12.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date

f. Lapsed time from sample collection to delivery
g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (~~at 7Q10 and~~ at time of sampling)
   f. Sample temperature when received at the laboratory
   g. Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source
   b. Collection date(s) and time(s)
   c. Pretreatment
   d. Physical and chemical characteristics

12.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food

12.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

12.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source

2. Date and time of most recent reference toxicant test, test results, and current control (cusum) chart
3. Dilution water used in reference toxicant test
4. Results (LC50 or where applicable, NOAEC)
5. Physical and chemical methods used

12.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50, NOAEC, Pass/Fail
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data

12.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

12.2 TEST REVIEW

12.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

12.2.2 SAMPLING AND HANDLING

12.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 12.1).

12.2.3 TEST ACCEPTABILITY CRITERIA

12.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

12.2.4 TEST CONDITIONS

12.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 12.1).

12.2.4.2 Deviations in test conditions (from the specifications listed in the summary of test condition tables) must be evaluated to determine the validity of test results. Test condition deviations may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The

reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid.

12.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

12.2.5 STATISTICAL METHODS

12.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 11.1.4), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

12.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

12.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

12.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

12.2.7 REFERENCE TOXICANT TESTING

12.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.15). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.15) and compared to the current control chart limits (± 2 standard deviations).

12.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.15). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

12.2.8 TEST VARIABILITY

12.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

12.2.8.2 In an effort to reduce the variability of WET test methods, USEPA guidance on WET variability recommends implementing upper and lower bounds on the PMSD calculated in a test for the sublethal endpoint (USEPA, 2000b). The minimum significant difference (MSD) is the smallest difference between the control and another test treatment that can be determined as statistically significant in a given test, and the PMSD is the MSD represented as a percentage of the control response. The equation and examples of MSD calculations are shown in Subsection 11.3.7.4.4.

12.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Replace Section 10, Report Preparation, on page 55 of the freshwater chronic method manual with the following.

SECTION 10
REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times

c. Sample collection method
d. Physical and chemical data
e. Streamflow (~~at 7Q10 and~~ at time of sampling)
f. Sample temperature when received at the laboratory
g. Lapsed time from sample collection to delivery

3. Dilution Water Samples
   a. Source
   b. Collection date(s) and time(s)
   c. Pretreatment
   d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test, test results, and current control (cusum) chart
3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc.
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 Deviations in test conditions (from the specifications listed in the summary of test condition tables) must be evaluated to determine the validity of test results. Test condition deviations may or may not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 In an effort to reduce the variability of WET test methods, USEPA guidance on WET variability recommends implementing upper and lower bounds on the PMSD calculated in a test for the sublethal endpoint (USEPA, 2000b). The minimum significant difference (MSD) is the smallest difference between the control and another test treatment that can be determined as statistically significant in a given test, and the PMSD is the MSD represented as a percentage of the control response. The equation and examples of MSD calculations are shown in Appendix C.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Replace Section 10, Report Preparation, on page 58 of the marine chronic method manual with the following.

SECTION 10
REPORT PREPARATION AND TEST REVIEW

10.1 REPORT PREPARATION

The following general format and content are recommended for the report:

10.1.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contract Laboratory (if the tests are performed under contract)
   a Name of firm
   b. Phone number
   c. Address

10.1.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)
8. Design flow of treatment facility at time of sampling

10.1.3 SOURCE OF EFFLUENT, RECEIVING WATER, AND DILUTION WATER

1. Effluent Samples
   a. Sampling point (including latitude and longitude)
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Mean daily discharge on sample collection date
   f. Lapsed time from sample collection to delivery
   g. Sample temperature when received at the laboratory

2. Receiving Water Samples
    a. Sampling point (including latitude and longitude)
    b. Collection dates and times
    c. Sample collection method
    d. Physical and chemical data
    e. Tide stages
    f. Sample temperature when received at the laboratory
    g. Lapsed time from sample collection to delivery

3. Dilution Water Samples
    a. Source
    b. Collection date(s) and time(s)
    c. Pretreatment
    d. Physical and chemical characteristics

10.1.4 TEST METHODS

1. Toxicity test method used (title, number, source)
2. Endpoint(s) of test
3. Deviation(s) from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type and volume of test chambers
7. Volume of solution used per chamber
8. Number of organisms per test chamber
9. Number of replicate test chambers per treatment
10. Acclimation of test organisms (temperature and salinity mean and range)
11. Test temperature (mean and range)
12. Specify if aeration was needed
13. Feeding frequency, and amount and type of food
14. Test salinity (mean and range)
15. Specify if (and how) pH control measures were implemented

10.1.5 TEST ORGANISMS

1. Scientific name and how determined
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)
7. Taxonomic key used for species identification

10.1.6 QUALITY ASSURANCE

1. Reference toxicant used routinely; source
2. Date and time of most recent reference toxicant test, test results, and current control (cusum) chart

3. Dilution water used in reference toxicant test
4. Results (NOEC or, where applicable, LOEC, LC50, EC50, IC25 and/or IC50); report percent minimum significant difference (PMSD) calculated in reference toxicant test
5. Physical and chemical methods used

10.1.7 RESULTS

1. Provide raw toxicity data in tabular form, including daily records of affected organisms in each concentration (including controls) and replicate, and in graphical form (plots of toxicity data)
2. Provide table of LC50s, NOECs, IC25, IC50, etc.
3. Indicate statistical methods used to calculate endpoints
4. Provide summary table of physical and chemical data
5. Tabulate QA data
6. Provide percent minimum significant difference (PMSD) calculated for sublethal endpoints

10.1.8 CONCLUSIONS AND RECOMMENDATIONS

1. Relationship between test endpoints and permit limits
2. Actions to be taken

10.2 TEST REVIEW

10.2.1 Test review is an important part of an overall quality assurance program (Section 4) and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority.

10.2.2 SAMPLING AND HANDLING

10.2.2.1 The collection and handling of samples are reviewed to verify that the sampling and handling procedures given in Section 8 were followed. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times (Subsection 8.5.4). Any deviations from the procedures given in Section 8 should be documented and described in the data report (Subsection 10.1).

10.2.3 TEST ACCEPTABILITY CRITERIA

10.2.3.1 Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample.

10.2.4 TEST CONDITIONS

10.2.4.1 Test conditions are reviewed and compared to the specifications listed in the summary of test condition tables provided for each method. Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) also are reviewed and compared to specified ranges. Any deviations from specifications should be documented and described in the data report (Subsection 10.1).

10.2.4.2 Deviations in test conditions (from the specifications listed in the summary of test condition tables) must be evaluated to determine the validity of test results. Test condition deviations may or may

not invalidate a test result depending on the degree of the departure and the objective of the test. The reviewer should consider the degree of the deviation and the potential or observed impact of the deviation on the test result before rejecting or accepting a test result as valid.

10.2.4.3 Whereas slight deviations in test conditions may not invalidate an individual test result, test condition deviations that continue to occur frequently in a given laboratory may indicate the need for improved quality control in that laboratory.

10.2.5 STATISTICAL METHODS

10.2.5.1 The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report. Statistical methods other than those recommended in the statistical flowcharts may be appropriate (see Subsection 9.4.1.2), however, the laboratory must document the use of and provide the rationale for the use of any alternate statistical method. In all cases (flowchart recommended methods or alternate methods), reviewers should verify that the necessary assumptions are met for the statistical method used.

10.2.6 CONCENTRATION-RESPONSE RELATIONSHIPS

10.2.6.1 The concept of a concentration-response, or more classically, a dose-response relationship is "the most fundamental and pervasive one in toxicology" (Casarett and Doull, 1975). This concept assumes that there is a causal relationship between the dose of a toxicant (or concentration for toxicants in solution) and a measured response. A response may be any measurable biochemical or biological parameter that is correlated with exposure to the toxicant. The classical concentration-response relationship is depicted as a sigmoidal shaped curve, however, the particular shape of the concentration-response curve may differ for each coupled toxicant and response pair. In general, more severe responses (such as acute effects) occur at higher concentrations of the toxicant, and less severe responses (such as chronic effects) occur at lower concentrations. A single toxicant also may produce multiple responses, each characterized by a concentration-response relationship. A corollary of the concentration-response concept is that every toxicant should exhibit a concentration-response relationship, given that the appropriate response is measured and given that the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results.

10.2.6.2 The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. USEPA (2000a) provides guidance on evaluating concentration-response relationships to assist in determining the validity of WET test results. All WET test results (from multi-concentration tests) reported under the NPDES program should be reviewed and reported according to USEPA guidance on the evaluation of concentration-response relationships (USEPA, 2000a). This guidance provides review steps for 10 different concentration-response patterns that may be encountered in WET test data. Based on the review, the guidance provides one of three determinations: that calculated effect concentrations are reliable and should be reported, that calculated effect concentrations are anomalous and should be explained, or that the test was inconclusive and the test should be repeated with a newly collected sample. It should be noted that the determination of a valid concentration-response relationship is not always clear cut. Data from some tests may suggest consultation with professional toxicologists and/or regulatory officials. Tests that exhibit unexpected concentration-response relationships also may indicate a need for further investigation and possible retesting.

10.2.7 REFERENCE TOXICANT TESTING

10.2.7.1 Test review of a given effluent or receiving water test should include review of the associated reference toxicant test and current control chart. Reference toxicant testing and control charting is required for documenting the quality of test organisms (Subsection 4.7) and ongoing laboratory performance (Subsection 4.16). The reviewer should verify that a quality control reference toxicant test was conducted according to the specified frequency required by the permitting authority or recommended by the method (e.g., monthly). The test acceptability criteria, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was a valid test. The results of the reference toxicant test are then plotted on a control chart (see Subsection 4.16) and compared to the current control chart limits (± 2 standard deviations).

10.2.7.2 Reference toxicant tests that fall outside of recommended control chart limits are evaluated to determine the validity of associated effluent and receiving water tests (see Subsection 4.16). An out of control reference toxicant test result does not necessarily invalidate associated test results. The reviewer should consider the degree to which the reference toxicant test result fell outside of control chart limits, the width of the limits, the direction of the deviation (toward increasing test organism sensitivity or toward decreasing test organism sensitivity), the test conditions of both the effluent test and the reference toxicant test, and the objective of the test. More frequent and/or concurrent reference toxicant testing may be advantageous if recent problems (e.g., invalid tests, reference toxicant test results outside of control chart limits, reduced health of organism cultures, or increased within-test variability) have been identified in testing.

10.2.8 TEST VARIABILITY

10.2.8.1 The within-test variability of individual tests should be reviewed. Excessive within-test variability may invalidate a test result and warrant retesting. For evaluating within-test variability, reviewers should consult EPA guidance on upper and lower percent minimum significant difference (PMSD) bounds (USEPA, 2000b).

10.2.8.2 In an effort to reduce the variability of WET test methods, USEPA guidance on WET variability recommends implementing upper and lower bounds on the PMSD calculated in a test for the sublethal endpoint (USEPA, 2000b). The minimum significant difference (MSD) is the smallest difference between the control and another test treatment that can be determined as statistically significant in a given test, and the PMSD is the MSD represented as a percentage of the control response. The equation and examples of MSD calculations are shown in Appendix C.

10.2.8.3 To assist in reviewing within-test variability, EPA recommends maintaining control charts of PMSDs calculated for successive effluent tests (USEPA, 2000b). A control chart of PMSD values characterizes the range of variability observed within a given laboratory, and allows comparison of individual test PMSDs with the laboratory's typical range of variability. Control charts of other variability and test performance measures, such as the MSD, standard deviation or CV of control responses, or average control response, also may be useful for reviewing tests and minimizing variability.

VII. Nominal Error Rates

A. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 9.4.6, Recommended Alpha Levels, on page 49 of the freshwater chronic method manual.

9.4.6.2 Under specific circumstances, the alpha level used for hypothesis testing may be reduced from 0.05 to 0.01. These circumstances apply when sublethal endpoints from *Ceriodaphnia dubia* or fathead minnow tests are reported under NPDES permit requirements, or when WET permit limits are derived without allowing for receiving water dilution. Even under these specific circumstances, the alpha level in a test may only be reduced if adequate test sensitivity can be maintained. USEPA guidance on nominal error rate adjustments (USEPA, 2000a) provides procedures for determining adequate test sensitivity and for determining the appropriateness of reductions in the alpha level.

B. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 9.4.6, Recommended Alpha Levels, on page 52 of the marine chronic method manual.

9.4.6.2 Under specific circumstances, the alpha level used for hypothesis testing may be reduced from 0.05 to 0.01. These circumstances apply when WET permit limits are derived without allowing for receiving water dilution. Even under these specific circumstances, the alpha level in a test may only be reduced if adequate test sensitivity can be maintained. USEPA guidance on nominal error rate adjustments (USEPA, 2000a) provide procedures for determining adequate test sensitivity and for determining the appropriateness of reductions in the alpha level.

VIII. Confidence Intervals

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Insert the following into Subsection 11.2, Determination of the LC50 from Definitive, Multi-effluent-concentration Acute Toxicity Tests, on page 76 of the acute method manual.

11.2.1.2 The Probit Method, the Spearman-Karber Method, and the Trimmed Spearman-Karber Method are designed to produce LC50 values and associated 95% confidence intervals. It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 9.3.2, Point Estimation Techniques, on page 47 of the freshwater chronic method manual.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 9.3.2, Point Estimation Techniques, on page 50 of the marine chronic method manual.

9.3.2.2 It should be noted that software used to calculate point estimates occasionally may not provide associated 95% confidence intervals. This situation may arise when test data do not meet specific assumptions required by the statistical methods, when point estimates are outside of the test concentration range, and when specific limitations imposed by the software are encountered. USEPA (2000a) provides guidance on confidence intervals under these circumstances.

IX. Dilution Series

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Insert the following into Subsection 9.3, Multi-concentration (Definitive) Effluent Toxicity Tests, on page 47 of the acute method manual.

9.3.2.1 USEPA recommends the use of a ≥0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 8.10, Multi-concentration (Definitive) Effluent Toxicity Tests, on page 42 of the freshwater chronic method manual.

8.10.2.1 USEPA recommends the use of a ≥0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 8.10, Multi-concentration (Definitive) Effluent Toxicity Tests, on page 45 of the marine chronic method manual.

8.10.2.1 USEPA recommends the use of a ≥0.5 dilution factor for selecting effluent test concentrations. Effluent test concentrations of 6.25%, 12.5%, 25%, 50%, and 100% are commonly used, however, test concentrations should be selected independently for each test based on the objective of the study, the expected range of toxicity, the receiving water concentration, and any available historical testing information on the effluent. USEPA (2000a) provides additional guidance on choosing appropriate test concentrations.

X. Dilution Waters

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Insert the following into Subsection 7.1, Types of Dilution Water, on page 33 of the acute method manual.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

B. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 7.1, Types of Dilution Water, on page 31 of the freshwater chronic method manual.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

C. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 7.1, Types of Dilution Water, on page 32 of the marine chronic method manual.

7.1.2 An acceptable dilution water is one which is appropriate for the objectives of the test; supports adequate performance of the test organisms with respect to survival, growth, reproduction, or other responses that may be measured in the test (i.e., consistently meets test acceptability criteria for control responses); is consistent in quality; and does not contain contaminants that could produce toxicity. USEPA (2000a) provides additional guidance on selecting appropriate dilution waters.

XI. Pathogen Interference

A. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Replace Subsection 11.3.4 on page 59 of the freshwater chronic method manual with the following.

11.3.4 Pathogenic and/or predatory organisms in ~~the dilution water and effluent~~ effluent samples or receiving water that is used for dilution may affect test organism survival and confound test results. A typical indication that pathogen interference has occurred in a WET test is when test organisms exhibit "sporadic mortality". This sporadic morality phenomenon is characterized by an unexpected concentration-response relationship (i.e., effects that do not increase with increasing effluent concentration) and organism survival that varies greatly among replicates and among effluent dilutions (USEPA, 2000a). The observed sporadic mortality among replicates may occur in receiving water controls and lower effluent concentrations on day 3 or day 4 of the chronic test and occasionally in the full-strength effluent.

11.3.4.1 Observations of sporadic mortality must be documented. When sporadic mortality occurs, a fungal growth may appear directly on the fish, especially in the gill area. The fungal growth has been attributed to *Saprolegnia sp.* (Downey *et al.*, 2000). Microbiological evaluations on receiving waters, the fish, and the food indicated the ubiquitous nature of pathogenic organisms (e.g., *Flexibacter spp*., *Aeromonas hydrophila*), and eradicating them from the test through the decontamination of the fish and their food has not been practical (Geis *et al.*, 2000).

11.3.4.2 When pathogen interference is suspected, a series of data evaluations are required. The test data must be reviewed to determine a cause for any unexpected concentration-response pattern and subsequently to determine the validity of calculated results (USEPA, 2000a). Normal, reversed, or bimodal dose response curves are not considered indicators of test interference by pathogenic bacteria (USEPA, 2000a). Each treatment (including the control) should be evaluated for an unusually high mortality response and unevenness of mortalities among replicates. If the within-treatment coefficient of variation (CVs) for survival in an effluent treatment is >40% but relatively small for control replicates in a standard reconstituted water, pathogen interference should be considered. Receiving water controls from improper preparation or collection also should be evaluated.

11.3.4.3 Because of the ubiquitous nature of the pathogens or predatory organisms, all test equipment, glassware, and pipettes must be kept clean and dry when not in use. Use of separate glassware, pipettes, and siphons for each concentration is recommended to minimize cross contaminating replicates of all treatments.

11.3.4.4 When filtration through a 2-4 mm mesh opening (Subsection 8.8.2) and proper laboratory hygiene (Subsection 11.3.4.3) do not reduce the interference, the analyst will need to determine if the toxicity test results could be due to pathogens. Parallel tests should be conducted using reconstituted water and receiving water as diluents with the effluent to confirm that the test results are due to pathogen interference. When the effluent exhibits the interference, both the intake water and effluent samples must be tested to determine the source of pathogens and to determine if the intake water is contributing the interference observed in the toxicity test of the final effluent. When the dilution water exhibits the interference, reconstituted laboratory water instead of receiving waters should be used to minimize the

interference. However, if receiving water is required, modify the test design to minimize the effects of the interference (Subsection 11.3.4.5) prior to treating the receiving water diluent or effluent sample (Subsection 11.3.4.6).

11.3.4.5 When data evaluation indicates that sporadic mortality has occurred as described in Subsection 11.3.4, the test design can be modified as described below to minimize pathogen interference. The use of 2 fish per 20 ml in each 1 ounce plastic cup test solution or 2 fish per 50 ml in each 4 ounce plastic cup can be used rather than 10 fish per test chamber. The total number of fish tested remains unchanged (i.e., 40 per treatment). At test initiation, for each test concentration and replicate, the test cups must be labeled to easily recombine the fish to the original replicate at the end of the test. For example, for replicate A, each of the five plastic test cups would be identified as subreplicate A1, A2, A3, A4, and A5 repeating the pattern for subsequent replicates (e.g., for replicate B, each cup would be identified as subreplicate B1, B2, B3, B4, and B5). At test termination, all test organisms from the five A subreplicates are combined for a survival and weight determination. Document the recombination of replicates in records.

11.3.4.5.1 All test chambers must be randomized using a template for randomization or by using a table of random numbers. For either a randomized or randomized block design (see Subsection 13.10.2), test chambers are randomized once at the beginning of the test. When using templates, a number of different templates should be prepared, so that the same template is not used for every test. Randomization procedures must be documented with daily records.

11.3.4.5.2 When adding or transferring the larvae to test chambers, the amount of excess water added to the chambers should be kept to a minimum to avoid unnecessary dilution of the test concentrations. The fish in each test chamber should be fed 0.1 ml of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4 h intervals, or 0.15 ml should be fed twice daily at an interval of 6 h. (NOTE: to prevent low dissolved oxygen levels, the amount of food added to cups should be adjusted to account for the modified test design that uses smaller test chambers). Dead test organisms should be removed as soon as they are observed.

11.3.4.5.3 Fish are transferred to new or clean test chambers daily. At the time of the daily renewal of the test solutions, the fish are transferred to a new test chamber containing fresh test solution using a pipette which has at least a 5mm bore diameter. Separate pipettes should be used for each treatment. Water transfer is kept to a minimum by allowing the fish to swim out of the pipette into the new test chamber. Any potential injury to individual fish should be recorded on the test sheets.

11.3.4.5.4 At test termination, the surviving larvae in each chamber must be counted and all subreplicates within a replicate (e.g., A1, A2, A3, A4, and A5) combined. For example, all test cups (within a treatment) labeled A would be combined for a survival and dry weight determination.

11.3.4.6 When parallel testing has confirmed pathogen interference and the above test design modification (Section 11.3.4.5) does not reduce the pathogen interference, the regulatory authority may allow modifications of the effluent samples or receiving water diluent to remove or inactivate the pathogens. TIE approaches (USEPA, 1991b; USEPA, 1992) and various sterilization techniques can be applied to assess the source of the sporadic mortality. The following procedures can be used alone or in combination to ascertain the adverse influence on tests caused by pathogens in the intake or receiving waters used for dilution. The effects of pathogenic bacteria must be confirmed by parallel and simultaneous testing of the following procedures with altered and unaltered samples.

11.3.4.6.1 Use of ultra-violet light to irradiate the sample. The rate of pumping specified by the manufacturer of the apparatus should be used, and the life of the UV light source must follow manufacturers' recommendations and be documented. For example, one liter of water can be irradiated for 20 min using an 8 watt UV light (Aquatic Ecosystems, Apopka, FL) prior to use each day of the test. Light sources have limited lifetimes and their effectiveness will decrease with age. The delivery pump and the light source should be on the same electrical circuit to ensure that when power is interrupted both terminate operation. QA/QC procedures should be put into place to assure that the light source is on at the beginning and at the end of the procedure. Treatment of the large volumes of water necessary for test dilution also may be impractical. Caution: Since the effluent or receiving water samples must be passed through the UV sterilizer and then test treatments prepared, there may be potential effects of UV light on the sample. UV exposure may increase or decrease toxicity from other pollutants in the sample. UV treatment is known to cause photoactivation of some organic compounds, which may increase toxicity. UV treatment also is known to cause the photochemical breakdown of certain organic compounds, which could decrease toxicity (if the parent compound is toxic) or increase toxicity (if reaction products are toxic). The effectiveness of UV for sterilization may decrease with turbid or stained samples. Bacteria can escape exposure by being lodged in crevices of particulate matter in the sample. All toxicity tests using a sterilized sample must include a blank preparation consisting of similarly sterilized laboratory water.

11.3.4.6.2 Ultra-filtration through a 0.22 µm pore diameter filter (such as Gelman Suprocap®) may be conducted on sample aliquots before daily use. Samples may need to be filtered through a glass fiber filter prior to the 0.22 µm filter. This is time consuming and volume restricted. Treatment of the large volumes of water necessary for test dilution may be impractical. Caution: Since the effluent or receiving water samples must be passed through the filter, the effect of filtering must be evaluated. Filtration can remove toxicity if toxic components of the sample are bound to particles (USEPA, 1991b; 1992). The removal of suspended solids also may influence the bioavailability of chemical pollutants. The removal of toxicity by filtration must be evaluated for each sample by testing samples before and after filtration. All toxicity tests using a sterilized sample also must include a blank preparation consisting of similarly sterilized reconstituted laboratory water.

11.3.4.6.3 Use of chlorination and dechlorination. In some cases, pathogens can survive the chlorination/dechlorination process and the pathogenic effects may increase due to lack of competition from other organisms. Sufficient data must be collected and documented to determine the effective dosage required. Toxicity tests conducted with the addition of chlorine and subsequent dechlorination (USEPA, 1991b; 1992) to either effluent or receiving water samples also must include a blank preparation consisting of similarly treated laboratory water.

11.3.4.6.4 Use of antibiotics. The addition of wide spectrum antibiotics has been effective in removing the pathogen effect (Downey *et al.*, 2000). Antibacterial treatment such as those commonly used in aquaculture or home aquarium maintenance (e.g., oxytetracycline, chloramphenicol, and actinomycin) may be effective. Sufficient data must be collected to determine the effective dosage required. Caution: While antibiotics are effective, easy to use, inexpensive, and readily available, the antibiotic treatment may alter the sample in unknown or undesirable ways and may make the sample too cloudy. Large volumes of a sample may need to be treated. All toxicity tests using antibiotic treatments also must include treatment blanks of similarly prepared laboratory water.

XII. *Selenastrum capricornutum* Growth Test Method

A. Freshwater Chronic Method

The following are proposed changes to the freshwater chronic method manual.

1. Replace Subsection 14.6.16.2 on page 199 of the freshwater chronic method manual with the following.

14.6.16.2 Algal Culture Medium is prepared as follows:

14.6.16.2.1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1. ~~Cautionary note : EDTA may affect metal toxicity. It is recommended that tests be conducted with and without EDTA in the culture media if metals are suspected in the effluent or receiving water.~~

14.6.16.2.2. Add 1mL of each stock solution, in the order listed in Table 1, ...

14.6.16.2.3. Immediately filter the pH-adjusted medium through a 0.45 µm pore....

14.6.16.2.4. If the filtration is carried out with sterile apparatus, ...

14.6.16.2.5. Unused sterile medium should not be stored more than one week....

14.6.16.2.6 When prepared according to Table 1, the micronutrient stock solution contains ethylenediaminetetraacetic acid (EDTA). This nutrient stock formulation containing EDTA is recommended for culturing and testing with *Selenastrum capricornutum*. EDTA should be included in the nutrients added to algal culture media, test dilution water (see Subsection 14.10.1.3.1), and samples prior to testing (see Subsection 14.10.1.2.7). The use of EDTA improves test method performance by reducing the incidence of false positives and increasing test method precision. In interlaboratory testing of split samples analyzed with and without the addition of EDTA, false positive rates were 0.00% with EDTA and 33.3% without EDTA (USEPA, 2001a). Interlaboratory variability, expressed as the CV for IC25 values, was 34.3% with EDTA and 58.5% without EDTA (USEPA, 2001a). While the use of EDTA is recommended, testing without the addition of EDTA may be appropriate in some cases. EDTA effectively binds some metals, so the use of EDTA in the test procedure may cause tests to underestimate toxicity if metals are the primary contributor to sample toxicity. In cases where metals are known to contribute to sample toxicity, testing without the addition of EDTA may be conducted if the testing laboratory has demonstrated success in the use of the without EDTA procedure. Demonstrated success should include documentation of meeting appropriate test acceptability criteria and control charts of reference toxicant tests conducted without the addition of EDTA.

2. Replace Subsection 14.12, Acceptability of Test Results, on page 209 of the freshwater chronic method manual with the following.

14.12 ACCEPTABILITY OF TEST RESULTS

14.12.1 For the test results to be acceptable, the mean algal cell density in the control flasks must exceed 1 X 10^6 cells/mL ~~with EDTA or 2 X 10^5 cells/mL without EDTA~~ at the end of the test, and ~~not vary more than 20% among replicates~~ the coefficient of variation (CV, calculated as standard deviation X 100 / mean) for algal cell density among the control replicates must not exceed 20%.

14.12.2 If the test is conducted without including EDTA in the nutrient stock solution that is added to the dilution water and sample (see Subsection 14.6.16.2.6), the test acceptability criteria for mean algal cell density may be reduced. For the results of tests conducted without EDTA to be acceptable, the mean algal cell density in the control flasks must exceed 2 X 10^5 cells/mL at the end of the test, and the CV (calculated as standard deviation X 100 / mean) for algal cell density among the control replicates must not exceed 20%.

3. Replace Table 3 (continued) on page 211 of the freshwater chronic method manual with the following.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GREEN ALGA, *SELENASTRUM CAPRICORNUTUM*, GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

14.	Test concentrations:	Effluents: Minimum of 5 and a control Receiving Water: 100% receiving water or minimum of 5 and a control
15.	Test dilution factor:	Effluents: ≥0.5 Receiving Waters: None or ≥0.5
16.	Test duration:	96 h
17.	Endpoint:	Growth (cell counts, chlorophyll fluorescence, absorbance, biomass)
18.	Test acceptability criteria:[2]	Mean cell density of at least 1 X 10^6 cells/mL ~~with EDTA or 2 X 10^5 cells/mL without EDTA~~ in the controls~~:~~; and ~~V~~variability (CV%) among ~~of~~ control~~s~~ replicates less than or equal to ~~should not exceed~~ 20%
19.	Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)
20.	Sample volume required:	1 or 2 L depending on test volume

[2] If the test is conducted without including EDTA in the nutrient stock solution that is added to the dilution water and the sample, the test acceptability criteria are a mean cell density of at least 2 X 10^5 cells/mL in the controls, and variability (CV%) among control replicates less than or equal to 20%.

XIII. *Mysidopsis bahia* Survival, Growth, and Fecundity Test Method

A. Marine Chronic Method

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 14.6.13.2, Test Organisms, on page 233 of the marine chronic manual.

14.6.13.2.11 The pre-test holding conditions of test organisms (as well as the test conditions) have been shown to significantly influence the success of achieving the test acceptability criteria for the fecundity endpoint (egg production by 50% or more of control females). Temperature, feeding, and organism density are important factors in the rate of mysid development. Laboratories should optimize these factors (within the limits of the test procedure) during both the pre-test holding period and the testing period to encourage achieving the test acceptability criteria for the fecundity endpoint. If test organisms are purchased, the testing laboratory should also confer with the supplier to ensure that pre-test holding conditions are optimized to successfully achieve the fecundity endpoint. Lussier *et al.* (1999) found that by increasing holding temperature and test temperature from 26˚C ± 1˚C to 26˚C - 27˚C and maintaining holding densities to ≤10 organisms / L, the percentage of tests meeting the test acceptability criteria for fecundity increased from 60% to 97%. While the fecundity endpoint is an optional endpoint, it is often the most sensitive measure of toxicity, and the 7-d mysid test estimates the chronic toxicity of effluents most effectively when all three endpoints (survival, growth, and fecundity) are measured (Lussier *et al.* 1999).

XIV. *Holmesimysis costata* Acute Test Method

A. Acute Method Manual

The following are proposed changes to the acute method manual.

1. Replace Subsections 6.1.2 and 6.1.3 on page 27 of the acute method manual with the following.

6.1.2 Toxicity test conditions and culture methods are provided in this manual for the following principal test organisms:

Freshwater Organisms:
1. *Ceriodaphnia dubia* (daphnid) (Table 11).
2. *Daphnia pulex* and *D. magna* (daphnids) (Table 12).
3. *Pimephales promelas* (fathead minnow) (Table 13).
4. *Oncorhynchus mykiss* (rainbow trout) and *Salvelinus fontinalis* (brook trout) (Table 14).

Estuarine and Marine Organisms:

1. *Mysidopsis bahia* (mysid) (Table 15).[1]
2. *Cyprinodon variegatus* (sheepshead minnow) (Table 16).
3. *Menidia beryllina* (inland silverside), *M. menidia* (Atlantic silverside), and *M. peninsulae* (tidewater silverside) (Table 17).
4. *Holmesimysis costata* (mysid) (Table 18).

6.1.3 The test species listed in Subsection 6.1.2 are the recommended acute toxicity test organisms. They are easily cultured in the laboratory, are sensitive to a variety of pollutants, and are generally available throughout the year from commercial sources. Summaries of test conditions for these species are provided in Tables 11-178. Guidelines for culturing and/or holding the organisms are provided in Appendix A.

2. Insert the following footnote on page 27 of the acute method manual.

[1] The genus name of this organism was formally changed to *Americamysis* (Price *et al.*, 1994), however, the method manual will continue to refer to *Mysidopsis bahia* to maintain consistency with previous versions of the method.

3. Replace Subsection 9.17, Summary of Test Conditions for the Principal Test Organisms, on page 56 of the acute method manual with the following.

9.17 SUMMARY OF TEST CONDITIONS FOR THE PRINCIPAL TEST ORGANISMS

9.17.1 Summaries of the test conditions for the daphnids, *Ceriodaphnia dubia*, *Daphnia pulex*, and *D. magna*, fathead minnows, *Pimephales promelas*, rainbow trout, *Oncorhynchus mykiss*, brook trout, *Salvelinus fontinalis*, the mysids, *Mysidopsis bahia* and *Holmesimysis costata*, sheepshead minnows, *Cyprinodon variegatus*, and silversides, *Menidia beryllina*, *M. menidia*, and *M. peninsulae*, are provided in Tables 11-178.

4. Replace Table 15 on page 65 of the acute method manual with the following.

TABLE 15. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR MYSID, *MYSIDOPSIS BAHIA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[~~‡~~]

1. Test type:	Static non-renewal, static-renewal, or flow-through
2. Test duration:	24, 48, or 96 h
3. Temperature:[~~2~~1]	20˚C ±1˚C; or 25˚C ±1˚C
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	250 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, after 48 h
10. Age of test organisms:	1-5 days; 24-h range in age
11. No. organisms per test chamber:	Minimum, 10 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 2 for effluent tests Minimum, 4 for receiving water tests
13. No. organisms per concentration:	Minimum, 20 for effluent tests Minimum, 40 for receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤24-h old, daily (approximately 100 nauplii per mysid)
15. Test chamber cleaning:	Cleaning not required

~~‡*Homesimysis costata* (mysid) can be used with the test conditions in this table, except at a temperature of 12˚C, instead of 20˚C or 25˚C, and a salinity of 32-34‰, instead of 5-30‰, where it is the required test organism in discharge permits.~~

~~2~~1 Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

5. Insert the following table after Table 17 on page 70 of the acute method manual.

TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS[1]

1. Test type:	Static non-renewal or static-renewal
2. Test duration:	24, 48, or 96 h
3. Temperature:	15˚C ± 1˚C for organisms collected South of Pt Conception, CA 13°C ± 1˚C for organisms collected North of Pt Conception, CA
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	10-20 μE/m^2/s (50-100 ft-c) (ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	1000 mL (minimum)
8. Test solution volume:	200 mL (minimum)
9. Renewal of test solutions:	Minimum, at 48 h
10. Age of test organisms:	3 to 4 days post-hatch juveniles
11. No. organisms per test chamber:	Minimum, 5 for effluent and receiving water tests
12. No. replicate chambers per concentration:	Minimum, 5 for effluent tests and receiving water tests
13. No. organisms per concentration:	Minimum, 25 for effluent tests and receiving water tests
14. Feeding regime:	*Artemia* nauplii are made available while holding prior to the test; feed 0.2 mL of concentrated suspension of *Artemia* nauplii ≤24-h old, daily approximately 40 nauplii per mysid)

[1] Acute and chronic toxicity tests performed simultaneously to obtain acute/chronic ratios must use the same temperature and salinity.

TABLE 18. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR WEST COAST MYSID, *HOLMESIMYSIS COSTATA*, ACUTE TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test chamber cleaning:	Cleaning not required
16. Test solution aeration:	None, unless DO concentration falls below 4.0 mg/L; rate should not exceed 100 bubbles/min
17. Dilution water:	34 ± 2‰ salinity; Uncontaminated seawater (1 µm filtered) or hypersaline brine or equivalent (see Section 7, Dilution Water)
18. Test concentrations:	Effluents: Minimum of five effluent concentrations and a control Receiving Waters: 100% receiving water and a control
19. Dilution series:	Effluents: ≥ 0.5 dilution series Receiving Waters: None, or ≥ 0.5 dilution series
20. Endpoint:	Effluents: Mortality (LC50 or NOAEC) Receiving Waters: Mortality (Significant difference from control)
21. Sampling and sample holding requirements:	Effluents and Receiving Waters: Grab or composite samples are used within 36 h of completion of the sampling period.
22. Sample volume required:	1 L for effluents 2 L for receiving waters
23. Test acceptability criterion:	90% or greater survival in controls

6. Replace the text of Appendix A.3 on page 172 of the acute method manual with the following.

APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.3. MYSIDS (*MYSIDOPSIS BAHIA* AND *HOLMESIMYSIS COSTATA*)

1. DISTRIBUTION

1.1 Mysids (Figure 1) are small shrimp-like crustaceans found in both the marine and freshwater environments. The mysid(s) that currently are ~~is~~ of primary interest in the NPDES program are ~~is~~ the estuarine species, *Mysidopsis bahia* (now identified as *Americamysis bahia;* Price *et al.*, 1994) and *Holmesimysis costata*.

1.2 *M. bahia* occurs primarily at salinities above 15‰; Stuck *et al.* (1979a) and Price (1982) found greatest abundances at salinities near 30‰. Three sympatric species of *Mysidopsis*, *M. almyra*, *M. bahia*, and *M. bigelowi*, have been cultured and used in toxicity testing. The distribution of *Mysidopsis* species has been reported by Stuck *et al.* (1979b), Price (1982), and Heard *et al.* (1987).

1.3 *H. costata* (Holmes 1900; previously referred to as *Acanthomysis sculpta*) is a west coast species that lives in the surface canopy of the giant kelp *Macrocystis pyrifera* where it feeds on zooplankters, kelp, epiphytes, and detritus. There are few references to the ecology of this mysid species (Holmquist, 1979; Clutter, 1967, 1969; Green, 1970; Turpen *et al.*, 1994). *H. costata* is numerically abundant in kelp forest habitats and is considered to be an important food source for kelp forest fish (Clark 1971, Mauchline 1980). *H. costata* eggs develop for about 20 days in their marsupium (abdominal pouch) before the young are released as juveniles; broods are released at night during molting. Females release their first brood at 55 to 70 days post-release (at 12˚C), and may have multiple broods throughout their approximately 120-day life.

1.4 ~~2~~ Other marine mysids that have been used in toxicity testing and held or cultured in the lab include *Metamysidopsis elongata*, *Neomysis americana*, *Neomysis awatschensis*, *Neomysis intermedia*, and recently for the Pacific coast, *Holmesimysis sculpta* and *Neomysis merce~~i~~dis*. A freshwater species, *Mysis relicta*, presently not used in toxicity testing, but found in the same habitat as *Daphnia pulex*, might be considered in the future for toxicity testing.

2. *MYSIDOPSIS BAHIA*

2.1 LIFE CYCLE

2.1.1 In laboratory culture, *Mysidopsis bahia* reach sexual maturity in 12 to 20 days, depending on water temperature and diet (Nimmo *et al.*, 1977). Normally, the female will have eggs in the ovary at approximately 12 days of age. The lamellae of the marsupium pouch have formed or are in the process of forming when the female is approximately 4 mm in length (Ward, 1993). Unlike *Daphnia*, the eggs will not develop unless fertilized. Mating takes place at night and lasts only a few minutes (Mauchline, 1980).

2.1.2 Brood pouches are normally fully formed at approximately 15 days (approximately 5 mm in body length), and young are released in 17 to 20 days (Ward, 1993). The number of eggs deposited in the

brood and the number of young produced per brood are a direct function of body length as well as environmental conditions. Mature females have produced as many as 25 Stage I larvae (egg-shaped embryo) per brood (8-9 mm in body length) in natural and artificial seawater (FORTY FATHOMS®) but average 11 ± 6 Stage III larvae (final stage before larvae are released), with increasing numbers correlated with increasing body length (Ward, 1993). A new brood is produced every 4 to 7 days.

2.1. 3 At time of emergence, juveniles are immobile, making them susceptible to predation by adult mysids. The juveniles are planktonic for the first 24-48 h and then settle to the bottom, orient to the current, and actively pursue food organisms such as *Artemia*. Carr *et al.* (1980) reported that the stage in the life cycle of *M. almyra* most sensitive to drilling mud was the juvenile molt, which occurs between 24 and 48 h after release from the brood pouch. Ward (1989) found a relationship between $CaCO_3$ level and growth and reproduction and that *M. bahia* were more sensitive to cadmium during molting (24-72 h post release) in high or low levels of $CaCO_3$. Work done by Lee and Buikema (1979) for *Daphnia pulex* also showed increased sensitivity during molting.

2.2~~3~~. MORPHOLOGY AND TAXONOMY

2.2.1 ~~3.1~~ Since *Mysidopsis bahia* occur with two other species of *Mysidopsis*, an understanding of the taxonomy of *M. almyra, M. bahia*, and *M. bigelowi* is important for culturing and testing practices. The taxonomic key of Heard *et al.* (1987) is suggested (see Table 1 for morphological guide to *Mysidopsis*).

2.2.2 ~~3.2~~ Adults of *M. bahia* range in length from 4.4 mm to 9.4 mm (Molenock, 1969), measured from the anterior margin of the carapace to the end of uropods. The mature females are normally larger than the males and the pleopods of the female are smaller than those of the male (Ward, 1993) (Figure 2). *Mysidopsis bahia* can be positively identified as male or female when they are 4 mm in body length (Ward, 1993). Living organisms are usually transparent, but may be tinted yellow, brown or black. *Mysidopsis bigelowi* can be readily distinguished from *M. almyra* and *M. bahia* by the morphology of the second thoracic leg. *Mysidopsis bigelowi* has a greatly enlarged endopod of the thoracic limb 2 ("first leg") and the limb has a distinctive row of 6 to 12 spiniform setae on the inner margin of the sixth segment (Heard *et al.*, 1987). *Mysidopsis bahia* can also be distinguished from other species of *Mysidopsis* by the number of apical spines on the telson (4-5 pairs) and the number of spines on the inner uropods distal to the statocyst (normally 2-3) (Figure 2).

2.2.3 ~~3.3~~ Heard *et al.* (1987) state that the most reliable character for separating adult *M. almyra* and *M. bahia* is the number of spines on the inner uropods (*M. almyra* will always have a single spine). Further, Price (1982) found that for all stages of development for both species, the shape of the anterior margin of the carapace (rostral plate) could be used to distinguish *M. almyra* (broadly rounded) from *M. bahia* (more produced). Figure 2 illustrates the morphological features most useful in identifying *M. bahia* (redrawn Molenock, 1969; Heard *et al.*, 1987).

2.3 ~~4.1~~ CULTURE METHODS

2.3.1 ~~4.1~~ SOURCE OF ORGANISMS

2.3.1.1 ~~4.1.1~~ Starter cultures of mysids can be obtained from commercial sources, particularly in the Gulf of Mexico region for *M. almyra* and *M. bahia*.

2.3.1.2 ~~4.1.2~~ Mysids of different species can also be collected by plankton tows or dip nets (approximately 1.0 mm mesh size) in estuarine systems. Heard *et al.* (1987) have identified specimens of *M. bahia* along the eastern coast, however, it has been principally identified as a subtropical species

found in the Gulf of Mexico and along the east coast of Florida. Since many species of mysids may be present at a given collection site, the identification of the organisms selected for culture should be verified by an experienced taxonomist. The permittee should consult the permitting authority for guidance on the source of test organisms (indigenous or laboratory reared) before use.

2.4 ~~4.2~~ CULTURING SYSTEM

2.4.1 ~~4.2.1~~ Stock cultures can be maintained in continuous-flow or closed recirculating systems. In laboratory culture of *M. bahia*, recirculating systems are probably the most common practice. During the past ten years, a number of closed recirculating systems have been described (Nimmo *et al.*, 1978; Leger and Sorgeloos, 1982; Ward 1984; 1991). Since no single recirculating technique is the best in all respects, the system adopted will depend on the facilities and equipment available and the objectives of the culturing activities. Two other species of mysid, *M. almyra* and *M. bigelowi*, have also been successfully reared in the system described in this section (Ward, 1991). Further, there now exist a number of review papers (Venables, 1987 and Lussier *et al.*, 1988) that describe in detail techniques developed by others that will be very helpful in culturing *Mysidopsis*.

2.4.2 ~~4.2.2~~ Closed recirculating systems are unique because the re-used seawater they contain develops an unusual set of characteristics caused primarily by metabolic waste produced by the mysids. The accumulation of waste products and suspended particles in the water column is prevented by passing the seawater through a biological filtration system, in which ammonia and nitrite are oxidized by nitrifying bacteria.

2.5 ~~4.3~~ CULTURE TANKS

2.5.1 ~~4.3.1~~ Stock cultures of mysids are maintained in a closed recirculating system. The system should consist of four 200-L glass aquaria. However, smaller tanks, such as 80-L glass aquaria, can be used. When setting up a system, it is important to consider surface to volume ratio since this will determine how many mysids can be held in each aquarium. If smaller tanks must be used, the 20-gallon "high" form is recommended. Figure 3 (Ward, 1984; 1991) illustrates the main components of the biological filtration system. The flow rate through the filter is controlled by the water valve and is maintained between 4-5 L/min. This flow will be sufficient to establish a moderate current (from the filter return line) in the aquarium to allow the mysids (which are positively rheotactic) to align themselves with the current formed.

2.5.2 ~~4.3.2~~ The filtration system consists of commercially-available under-gravel filter plates and external power filter. Each aquarium has two filter plates, forming a false bottom on each side of the tank, on which 2 cm of crushed coral are placed. The external power filter (Eheim, model 2017) canister is layered as shown in Figure 3 with a thin layer of filter fiber between each layer of carbon and crushed oyster shells. There has been some modification of the original filtration system (Ward, 1984), with crushed coral instead of oyster shells used on the filter bed, because crushed coral does not dissolve in seawater as readily as crushed oyster shells. If the system described above cannot be used, an acceptable alternative is an airlift pumping arrangement (Spotte, 1979). Crushed coral and oyster shells are commercially available and should be washed with deionized water and autoclaved before use.

2.6 ~~4.4~~ CULTURE MEDIA

2.6.1 ~~4.4.1~~ A clean source of filtered natural seawater (0.45 μm pore diameter) should be used to culture *Mysidopsis bahia*, however, artificial seasalts (FORTY FATHOMS®) have also been successfully used (Ward, 1993). A salinity range between 20 and 30‰ can be used (25‰ is suggested) to culture *M. bahia*. Leger and Sorgeloos (1982) reported success in culturing *M. bahia* in a formula following

Dietrich and Kalle (Kalle, 1971), and still report continued use of this formula (Leger *et al.*, 1987b). Other commercial brands have also been used (Reitsema and Neff, 1980; Nimmo and Iley, 1982; Nimmo et al., 1988) with varying degrees of success. The culture methods presented in Ward (1984; 1991) have been tried with a number of commercial brands of artificial seawater listed in Bidwell and Spotte (1985). Commercial brands of seasalts can be extremely variable in the amount of $NaHCO_3$ they provide, which, if not controlled, can affect growth and reproduction (Ward; 1989, 1991). In a comparative study, Ward (1993) found normal larval development within the marsupium using both natural seawater and FORTY FATHOMS® (i.e., Stage I - embryo; Stage II - eyeless larva; Stage III - eyed larva which is the final stage before release) and stressed the importance of proper preparation of the seasalts and monitoring of conditions in the tank.

2.6.2 ~~4.4.2~~ The culture media should be aged to allow the build-up of nitrifying bacteria in the filter substrate. To expedite the aging process, 15 mL of a concentrated suspension of *Artemia* should be added daily. If using natural or artificial seawater, the carbonate alkalinity level should be maintained between 90 and 120 mg/L. It is also important to establish an algal community, *Spirulina subsalsa*, in the filter bed (Ward, 1984) and a healthy surface dwelling diatom community, *Nitzchia* sp., on the walls (Ward, 1991) in conjunction with the transfer of part of the biological filter from a healthy tank, when possible. After seven days, the suitability of the medium is checked by adding 20 adult mysids. If the organisms survive for 96 h, the culture should be suitable for stocking.

2.6.3 ~~4.4.3~~ If brine solutions are used, 100‰ salinity must not be exceeded. This corresponds to a carbonate alkalinity value of approximately 50 mg/L, which will allow relatively normal physiological mechanisms associated with $CaCO_3$ to occur during certain phases of the life cycle for *M. bahia* (Ward, 1989).

2.7 ~~4.5~~ ENVIRONMENTAL FACTORS

2.7.1 ~~4.5~~ Temperature must be maintained within a range of 24°C to 26°C. Twelve to sixteen h illumination should be provided daily at 50 to 100 ft-c. The daily light cycle can be provided by combining overhead room lights, cool-white fluorescent bulbs (approx. 50 ft-c, 12L:12D), with individual Grow-lux fluorescent bulbs placed horizontally over each tank (approx. 65 ft-c, 10L:14D). This procedure will avoid acute illumination changes by allowing the room lights to turn on 1 h before and 1 h after the aquaria lights. A timing device, such as an electronic microprocessor-based timer (ChronTrol®, model CD, or equivalent) can be used to control the light cycle. These procedures are fully outlined in Ward (1984; 1991).

2.7.2 ~~4.5.2~~ Good aeration (≥60% saturation by vigorous aeration with an air stone), a 10-20 percent exchange of seawater per week, and carbonate in the filtration system are essential in helping to control pH drops caused by oxidation of NH_4-N and NO_2-N by bacteria.

2.7.3 ~~4.5.3~~ The single most important environmental factor when culturing *Mysidopsis bahia* or other organisms in recirculators is the conversion of ammonia to nitrite, and nitrite to nitrate by nitrifying bacteria. Spotte (1979) has suggested upper limits of 0.1 mg total NH_4-N/L, 0.1 mg NO_2-N/L and 20 mg NO_3-N/L for good laboratory operation of recirculating systems. For the recirculating system and techniques described here for mysids, the levels of ammonia, nitrite and nitrate never exceeded 0.05 mg of total ammonia-N/L ($NH_{3(aq)}$and NH_4^+), 0.08 mg NO_2-N/L and 18 mg NO_3-N/L (Ward, 1991). The toxicity of ammonia is based primarily on unionized ammonia (NH_3) and the proportion of NH_3 species to NH_4^+ species is dependent on pH, ionic strength and temperature. It is strongly recommended that the concentrations of total ammonia, nitrite and nitrate do not exceed those reported here. The ammonia,

nitrite, and nitrate levels can be checked by using color comparison test kits such as those made by LaMotte Chemical or equivalent methods.

2.7.4 ~~4.5.4~~ Bacterial oxidation of excreted ammonia by two groups of autotrophic nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*), results in an increase of hydrogen ions, which causes a drop in pH and subsequent loss of buffering capacity. Typically, the culturist responds to the change in pH by adding Na_2CO_3 or $NaHCO_3$. However, such efforts to buffer against a drop in pH will result in an increase in alkalinity and the uncontrolled use of carbonates can affect reproduction, especially at higher alkalinity values (Ward; 1989, 1991). Therefore, when using carbonates to buffer against pH changes, alkalinity values should not exceed 120 mg/L, which is easily measured by using a titrator kit such as that available from LaMotte Chemical or equivalent methods.

2.7.5 ~~4.5.5~~ Figure 4 (from Ward, 1991) depicts juvenile production per aquarium, no buffer added, over a period of 24 weeks. A regression line was calculated for these data and the slope and correlation coefficient were analyzed by Student's t test. The data showed that even when the pH dropped as low of 7.5, there was a significant increase ($P < 0.001$) in juvenile production. However, the pH should be maintained above 7.8 by the controlled use of $NaHCO_3$ and frequent water exchanges.

2.8 ~~4.6~~ FEEDING

2.8.1 ~~4.6.1~~ Frequent feeding with live food is necessary to prevent cannibalism of the young by the adults. McKenny (1987) suggests feeding densities of 2-3 *Artemia* per mL of seawater and Lussier *et al.* (1988) suggest a feeding rate of 150 *Artemia* nauplii per mysid daily.

2.8.2 ~~4.6.2~~ In the *M. bahia-Artemia* predator-prey relationship, it is also important to provide sufficient quantities of nutritionally viable free-swimming stage-I nauplii (Ward, 1987); final hatching from the membranous-sac (pre-nauplii) into stage-I nauplii does not always occur. *Artemia* cysts that have been incubated for 24 h should be periodically examined with a stereozoom microscope to enumerate free-swimming stage-I nauplii and prenauplii (membranous-sac stage).

2.8.3 ~~4.6.3~~ It has also been found that heavy metals can affect the hatchability of *Artemia* (Rafiee *et al.*, 1986; Liu and Chen, 1987), therefore, when using natural seawater the level of metals should always be checked.

2.8.4 ~~4.6.4~~ Ward (1987; 1991) has tried different brands of *Artemia* from different geographic origins and lot numbers; many achieved stage I nauplii and still caused variability in production of mysids which suggests that they were nutritionally lacking. Leger *et al.* (1985; 1987) have drawn attention to poor larval survival of *M. bahia* and low levels of certain polyunsaturated fatty acids found in the *Artemia* fed. The enhancement of *Artemia* has also been studied and there are numerous techniques that have been successful (Leger *et al.*, 1986).

2.8.5 ~~4.6.5~~ Ward (1987; 1991) has found that it is important to control the flow of seawater in recirculating systems (keep below 5 L/min) so that *Artemia* does not become limiting to the mysid. Newly hatched *Artemia* should be fed to mysids at least twice a day. To supply *Artemia* to the mysid population on the weekend and prevent cannibalism of newly released mysids, an automatic feeder such as described by Schimmel and Hansen (1975) or Ward (1984; 1991) could be used. Ward (1991) designed a system to hatch *Artemia* when personnel were not available to set up *Artemia* for the following morning and afternoon feeding, such as Monday. Cysts were placed in two 4-L Erlenmeyer flasks (dry), an airstone was placed in each flask, and two vessels overhead were filled with 3.5 L of

30‰ seawater each. The previously described timer (ChronTrol®, Model CD) was used to open the normally closed solenoids, allowing the seawater to gravity feed and hydrate the cysts.

2.8.6 ~~4.6.6~~ It is possible that a surface dwelling diatom community acts as a secondary food that supplements deficient brands of *Artemia*, especially for newly released juveniles. Ward (1991) has observed that a strong fertilizing action is caused by the excretory products of the mysid population. As the concentration of nitrate increases (nitrification) to about 5 mg/L (in approximately 7-10 weeks in an aquarium), a bloom of surface dwelling diatoms, principally *Nitzschia*, but including *Amphora* and *Cocconeis*, occurs in natural or artificial seawater (Ward, 1993). It is interesting to note that, at the same time, there is a dramatic increase in the number of juveniles observed in the aquaria (Figure 4). The diatoms form layers on the walls of the aquarium and swarms of newly released juveniles have been found among them, possibly feeding upon them.

2.8.7 ~~4.6.7~~ *Nitzschia* has been identified as a food source for the marine mud snail, *Ilyanassa obsoleta* (Collier, 1981), and the sea urchin, *Lytechinus pictus* (Hinegardner and Tuzzi, 1981). The diatom, *Skeletonema*, has also been used as a supplemental food for *M. bahia* (Venables, 1987). De Lisle and Roberts (1986) reported on the use of rotifers, *Branchionus plicatilis*, as a superior food for juvenile mysids. Rotifers are active swimmers, ranging in size from 100-175 μm as compared to 420-520 μm for *Artemia*, and would provide a good alternative food source if their fatty acid profile is adequate.

2.9 ~~4.7~~ CULTURE MAINTENANCE

2.9.1 ~~4.7.1~~ To avoid an excessive accumulation of algal growth on the internal surfaces of the aquaria, the walls and internal components should be scraped periodically and the shell substrate (coral or oyster) turned over weekly. Also, the filter plates must be completely covered so that the biological filter functions properly. After a culture tank has been in operation for approximately 2-3 months, detritus builds up on the bottom, which is removed with a fish net after first removing the mysids. The rate of water flow through the tanks should be maintained between 4-5 L/min, and 10-20% of the seawater in each aquarium should be exchanged weekly.

2.9.2 ~~4.7.2~~ Some culturists have noted problems with hydrozoan pests in their cultures and there are procedures for their eradication, if necessary (Lawler and Shepard, 1978; Hutton *et al.*, 1986).

2.10 ~~4.8~~ PRODUCTION LEVEL

2.10.1 ~~4.8.1~~ At least four aquaria should be maintained to insure a sufficient number of organisms on a continuing basis. If each 200-L aquarium is initially stocked with between 200 and 500 adults (do not exceed 500 adults), they will provide sufficient numbers of test organisms (Figure 4) each month. If the cultures are correctly maintained, at least 20 percent of the adult population should consist of gravid females (have a visible oostegite brood pouch with young). It is also advantageous to cull older mysids in the population every 4-6 weeks and to move mysids among the four aquaria to diversify the gene pool.

2.11 ~~5.~~ VIDEO TRAINING TAPE AVAILABLE FOR CULTURING METHODS

2.11.1 ~~5.1~~ A video training tape and supplemental report (USEPA, 1990) on culturing *Mysidopsis bahia* are available from the National AudioVisual Center, Customer Services Section, 8700 Edgeworth Drive, Capitol Heights, MD 20743-3701, (Phone 301-763-1891), as part of a video package on culturing and short-term chronic toxicity test methods (Order No. A18657; cost $75.00).

2.12 ~~6~~ TEST ORGANISMS

2.12.1 ~~6.1~~ Juvenile *Mysidopsis bahia*, one to five days old, are used in the acute toxicity test and the survival, growth and fecundity test (USEPA, 1994). To obtain the necessary number for a test, there are a number of techniques available. A mysid generator such as the one described by Reistsema and Neff (1980) has been successfully used. Another method to obtain juveniles is to take approximately 200 adult females (bearing embryos in their brood pouches) from the stock culture and place them in a large (10 cm X 15 cm) standard fish transfer net (2.0 to 3.0 mm openings) that is partially submerged in an 8-L aquarium containing 4 L of clean culture medium. As the juveniles are released from the brood pouches, they drop through the fish net into the aquarium. The adults and juveniles in the aquarium are fed twice daily 24-h post hydrated *Artemia*. The adults are allowed to remain in the net for 48 h, and are then returned to the stock tanks. The juveniles that are produced in the small tank may be used in the toxicity tests over a five-day period. Another method for obtaining juveniles (Ward 1987; 1989) is simply to remove juveniles from the stock culture with a fine mesh net, place them in 2-L Pyrex® crystalline dishes with media, positioned on a light table that has an attached viewing plate (2 mm squares), and remove juveniles less than 2 mm in length (approximately 24 h old).

3. *HOLMESIMYSIS COSTATA*

3.1 MORPHOLOGY AND TAXONOMY

3.1.1 Laboratories unfamiliar with the test organism should collect preliminary samples to verify species identification. Refer to Holmquist (1979) or send samples of mysids and any similar co-occurring organisms to a qualified taxonomist. Request certification of species identification from any organism supplier. Records of verification should be maintained along with a few preserved specimens. A review by Holmquist (1979) considered previous references to *Acanthomysis sculpta* in California to be synonymous with *Holmesimysis costata* and this is considered definitive at this time.

3.2 SOURCE OF BROODSTOCK AND TRANSPORT

3.2.1 Broodstock of *H. costata* are collected by sweeping a small-mesh (0.5-1 mm) hand net through the water just under the surface canopy blades of giant kelp *Macrocystis pyrifera*. Although this method collects mysids of all sizes, attention should be paid to the number of gravid females collected because these are used to produce the juvenile mysids used in toxicity testing. Gravid females are identified by their large, extended marsupia filled with young. Mysids should be collected from waters remote from sources of pollution to minimize the possibility of physiological or genetic adaptation to toxicants.

3.2.2 Mysids can be transported for a short time (< 3 h) in tightly covered 20 L plastic buckets. The buckets should be filled to the top with seawater from the collection site, and should be gently aerated or oxygenated to maintain dissolved oxygen above 60% saturation. Transport temperatures should remain within 3˚C of the temperature at the collection site.

3.2.3 For longer transport times of up to 36 h, mysids can be shipped in sealed plastic bags filled with seawater. The following transport procedure has been used successfully:

1) fill the plastic bag with one L of dilution water seawater,
2) saturate the seawater with oxygen by bubbling pure oxygen for at least 10 minutes,
3) place 25-30 adult mysids, or up to 100 juvenile mysids in each bag,
4) for adults add about 20 *Artemia* nauplii per mysid, for 100 juveniles add a pinch (10 to 20 mg) of ground Tetramin® flake food and 200 newly-hatched *Artemia* nauplii,
5) seal the bag securely, eliminating any airspace, and

6) place it within a second sealed bag in an ice chest.

Do not overfeed mysids in transport, as this may deplete dissolved oxygen, causing stress or mortality in transported mysids. A well-insulated ice chest should be cooled to approximately 15˚C by adding one 1-L blue ice block for every five 1-L bags of mysids (organisms will tolerate the temperature range of 12 to 16˚C). Wrap the ice in newspaper and a plastic bag to insulate it from the mysid bags. Pack the bags tightly to avoid shifting within the cooler.

3.3 HOLDING AND CULTURING

3.3.1 After collection, the mysids should be transported directly to the laboratory and placed in seawater tanks or aquaria equipped with flowing seawater or adequate aeration and filtration. Initial flow rates should be adjusted so that any temperature change occurs gradually (0.5˚C per h). Broodstock will be collected and maintained at two temperatures as follows: 1) maintain the water temperature of 15 ± 1˚C for mysids collected south of Pt. Conception, CA and 2) maintain the water temperatures of 13 ± 1˚C for mysids collected north of Pt. Conception, CA. Mysids can be cultured in tanks ranging from 4 to 1000 L. Tanks should be equipped with gentle aeration and blades of *Macrocystis* to provide habitat. Static culture tanks can be used if there is constant aeration, temperature control, and frequent water changes (one half the water volume changed at least twice a week). Maintain culture density below 20 animals per L by culling out adult males or juveniles.

3.4 FEEDING

3.4.1 Adult mysids should be fed 100 *Artemia* nauplii per mysid per day. Juveniles should be fed 5 to 10 newly released *Artemia* nauplii per juvenile per day and a pinch (10 to 20 mg) of ground Tetramin7 flake food per 100 juveniles per day. Static chambers should be carefully monitored and rations adjusted to prevent overfeeding and fouling of culture water.

3.5 TEST ORGANISMS

3.5.1 Juvenile *Holmesimysis costata* three to four days old, are used in the acute toxicity test and the survival and growth test (Hunt *et al.*, 1997; USEPA, 1995). To obtain the necessary number for a test, there are a number of techniques available for the acute toxicity test and the survival and growth test (USEPA, 1995). Approximately 150 gravid female mysids will typically produce approximately 400 juveniles. Gravid females can be identified by their large, extended marsupia filled with (visible) eyed juveniles. Marsupia appear distended and gray when females are ready to release young, due to presence of the juveniles. Gravid females are easily isolated from other mysids using the following technique: 1) use a small dip net to capture about 100 mysids from the culture tank, 2) transfer the mysids to a screen-bottomed plastic tube (150 µm-mesh, 25-cm diameter) partly immersed in a water bath or bucket, 3) lift the screen-tube out of the water to immobilize mysids on the damp screen, 4) gently draw the gravid females off the screen with a suction bulb and fire-polished glass tube (5-mm I.D.), and 5) collect gravid females in a separate screen tube. Re-immerse the screen continuously during the isolation process; mysids should not be exposed to air for more than a few seconds at a time.

3.5.2 Four to five days before a toxicity test begins, transfer gravid females into a removable, 2-mm-mesh screened cradle suspended within an aerated 80-L aquarium. Before transfer, make sure there are no juveniles in with the adult females. Extraneous juveniles are excluded to avoid inadvertently mixing them with the soon-to-be released juveniles used in testing. Provide the gravid females with newly hatched *Artemia* nauplii (approximately 200 per mysid) to help stimulate juvenile release. *Artemia* can be provided continuously throughout the night from an aerated reservoir holding approximately 75,000

Artemia. Direct the flow from the feeder into the screened compartment with the females, and add a few blades of *Macrocystis* for habitat. The females are placed within the screened compartment so that as the juveniles are released, they can swim through the mesh into the bottom of the aquarium. Outflows on flow-through aquaria should be screened (150-µm-mesh) to retain juveniles and allow some *Artemia* to escape.

3.5.3 Juveniles are generally released at night, so it is important to turn off all lights at night to promote release. In the morning, the screened compartment containing the females should be removed and placed in a separate aquarium. Juveniles should be slowly siphoned through a wide-diameter hose into a150-µm-mesh screen-bottom tube (25 cm diam.) immersed in a bucket filled with clean seawater. Once the release aquarium is emptied, it should be washed with hot fresh water to eliminate stray juveniles that might mix with the next cohort.

3.5.4 After collection, the number of juveniles should be estimated visually or by counting subsamples with a small beaker. If there are not enough juveniles, the juveniles from previous or subsequent releases can be combined so that the test is initiated with three and/or four-day old juveniles. Mysids 2-days old and younger have higher mortality rates, while mysids older than four days may vary in their toxicant sensitivity or survival rate (Hunt *et al.*, 1989; Martin *et al.*, 1989).

3.5.5 Test juveniles should be transferred to additional screen-tubes (or to 4-L static beakers if flowing seawater is unavailable). The screen-tubes are suspended in a 15-L bucket so that dilution water seawater (0.5 L/min) can flow into the tube, through the screen, and overflow from the bucket. Check water flow rates (< 1 L/min) to make sure that juveniles or *Artemia* nauplii are not forced down onto the screen. The height of the bucket determines the level of water in the screen tube. About 200 to 300 juveniles can be held in each screen-tube (200 juveniles per static 4-L beaker). Juveniles should be fed 40 newly hatched *Artemia* nauplii per mysid per day and a pinch (10 to 20 mg) of ground Tetramin7 flake food per 100 juveniles per day. A blade of *Macrocystis* (well rinsed in seawater) should be added to each chamber. Chambers should be gently aerated and temperature controlled at 15 ± 1°C (or 13 ± 1°C if collected north of Pt. Conception). Half of the seawater in static chambers should be changed at least once between isolation and test initiation.

3.5.6 The day juveniles are isolated is designated day 0 (the morning after their nighttime release). The toxicity test should begin on day three or four. For example, if juveniles are isolated on Friday, the toxicity test would begin on the following Monday or Tuesday. Pool all of the test juveniles into a 1-L beaker. Using a 10-mL wide-bore pipet or fire-polished glass tube (approximately 2-3 mm I.D), place one or two juveniles into as many plastic cups (one for each test chamber). These cups should contain enough clean dilution seawater to maintain water quality and temperature during the transfer process (approximately 50 mL per cup). When each of the cups contains one or two juveniles, repeat the process, adding mysids until each cup contains 10 organisms. Carefully pour or pipet off excess water in the cups, leaving less than 5 mL with the test mysids. This 5 mL volume can be estimated visually after initial measurements. Carefully pour or pipet the juveniles into the test chambers immediately after reducing the water volume. Gently rocking the water back and forth before pouring may help prevent juveniles from clinging to the walls of the randomization cups. Juveniles can become trapped in drops; have a squirt bottle ready to gently rinse down any trapped mysids. If more than 5 mL of water is added to the test solution with the juveniles, report the amount on the data sheet. Be sure that all water used in culture, transfer, and test solutions is within 1°C of the test temperature. Because of the small volumes involved in the transfer process, temperature control is best accomplished in a constant-temperature room.

3.5.7 Immobile mysids that do not respond to a stimulus are considered dead. The stimulus should be two or three gentle prods with a disposable pipet. Mysids that exhibit any response clearly visible to the naked eye are considered living. The most commonly observed movement in moribund mysids is a quick contraction of the abdomen. This or any other obvious movement qualifies a mysid as alive.

7. Insert the following references into the Selected References section of Appendix A.3 on page 184 of the acute method manual.

SELECTED REFERENCES

Clark, W. 1971. Mysids of the southern kelp region. In: W. North, ed., Biology of Giant Kelp Beds (Macrocystis) in California. J. Cramer Publisher, Lehre, Germany: p 369-380.

Clutter, R.I. 1967. Zonation of nearshore mysids. Ecology. 48(2): 200-208.

Clutter, R.I. 1969. The microdistribution and social behavior of some pelagic mysids. J. Exp. Mar. Biol. Ecol. 3: 125-155.

Green, J.M. 1970. Observations on the behavior and larval development of *Acanthomysis sculpta* (Tattersall), (Mysidacea). Can. J. Zool. 48: 289-292.

Holmquist, C. 1979. *Mysis costata* and its relations (Crustacea, Mysidacea). Zool. Jb. Syst. 106: 471-499.

Hunt, J.W., B.S. Anderson, S.L. Turpen, A.R. Coulen, M. Martin, F.H. Palmer, and J.J. Janik. 1989. Marine bioassay project fourth report: Experimental evaluation of effluent toxicity testing protocols with giant kelp, mysids, red abalone, and topsmelt. Report 89-5WQ. California State Water Resources Control Board, Sacramento, CA.

Hunt, J.W., B.S. Anderson, S.L.Turpen, M.A. Englund, and W.J. Piekarski. 1997. Precision and sensitivity of a seven-day growth and survival toxicity test using the west coast marine mysid crustacean *Holmesimysis costata*. Environ. Toxicol. Chem. 16: 824-834.

Martin, M., J.W. Hunt, B.S. Anderson, S.L. Turpen, and F.H. Palmer. 1989. Experimental evaluation of the mysid *Holmesimysis costata* as a test organism for effluent toxicity testing. Environ. Toxicol. Chem. 8: 1003-1012.

Turpen, S.L, J.W. Hunt, B.S. Anderson, and J.S. Pearse. 1994. Population structure, growth and fecundity of the kelp forest mysid, *Holmesmysis costata* in Monterey Bay, California. J. Crust. Biol. 14(4): 657-664.

USEPA. 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms, 2nd ed. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/4-91/003.

USEPA, 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH. EPA/600/R-95-136.

8. Replace footnote 2 in Appendix B on page 266 of the acute method manual with the following.

[2] Test conditions for *Holmesimysis costata* are found in Table 158

XV. Percent Minimum Significant Difference (PMSD)

A. Freshwater Chronic Method Manual

The following are proposed changes to the freshwater chronic method manual.

1. Insert the following into Subsection 11.13.1, General, on page 81 of the freshwater chronic method manual.

11.13.1.4 For hypothesis tests, the percent reduction from the control corresponding to the minimum significant difference (MSD) (see Subsection 9.6.1.4) is abbreviated herein as PMSD. Upper and lower bounds for the PMSD identified in USEPA (2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described in Subsections 11.13.3.7.13 and 11.13.3.7.14. The upper PMSD bound for the fathead minnow growth endpoint is 35% and the lower bound is 9.4%. The PMSD bounds are intended for use in determining the NOEC and LOEC in multi-concentration tests.

2. Insert the following into Subsection 11.13.3.7, Dunnett's Procedure, on page 102 of the freshwater chronic method manual.

11.13.3.7.11 Upper and lower bounds for the PMSD (USEPA, 2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described below in Subsections 11.13.3.7.13 and 11.13.3.7.14. The upper PMSD bound is 35% and the lower PMSD bound is 9.4%.

11.13.3.7.12 To make comparisons with upper and lower bounds for PMSD (USEPA, 2000b), calculate MSD as described in Appendix C, using Dunnett's critical value (d), and calculate the PMSD as described in Appendix C, Subsection 1.11.2.2. Calculate these whether or not the Dunnett's test will be used for hypothesis testing. The upper and lower bounds for PMSD (USEPA, 2000b) apply to untransformed growth data for a large sample of toxicity tests, including those in which Dunnett's test would not have been used for hypothesis testing. For the purposes of calculating PMSDs, use of Dunnett's test also is acceptable when there is variation among treatments in the number of replicates.

11.13.3.7.13 When the MSD represents a percent reduction from the control that is smaller than the lower PMSD bound for the growth endpoint of this test method, 9.4% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control smaller than 9.4%. These test concentrations will be considered as not differing significantly from the control for the purpose of determining the NOEC and LOEC.

11.13.3.7.14 When the MSD represents a percent reduction from the control that is larger than the upper PMSD bound for the growth endpoint of this test method, 35% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which do not differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control larger than 35%. These test concentrations will be considered as differing significantly from the control for the purpose of determining the NOEC and LOEC.

3. Insert the following into Subsection 13.13.1, General, on page 170 of the freshwater chronic method manual.

13.13.1.5 For hypothesis tests, the percent reduction from the control corresponding to the minimum significant difference (MSD) (see Subsection 9.6.1.4) is abbreviated herein as PMSD. Upper and lower bounds for the PMSD identified in USEPA (2000b) must be applied to the determination of NOEC and LOEC for the reproduction endpoint, as described in Subsections 13.13.3.7.13 and 13.13.3.7.14. The upper PMSD bound for the *Ceriodaphnia dubia* reproduction endpoint is 37%, and the lower bound is 11%. The PMSD bounds are intended for use in determining the NOEC and LOEC in multi-concentration tests.

4. Insert the following into Subsection 13.13.3.7, Dunnett's Procedure, on page 182 of the freshwater chronic method manual.

13.13.3.7.11 Upper and lower bounds for the PMSD (USEPA, 2000b) must be applied to the determination of NOEC and LOEC for the reproduction endpoint, as described below in Subsections 13.13.3.7.13 and 13.13.3.7.14. The upper PMSD bound is 37% and the lower PMSD bound is 11%.

13.13.3.7.12 To make comparisons with upper and lower bounds for PMSD (USEPA, 2000b), calculate MSD as described in Appendix C, using Dunnett's critical value (d), and calculate the PMSD as described in Appendix C, Subsection 1.11.2.2. Calculate these whether or not the Dunnett's test will be used for hypothesis testing. The upper and lower bounds for PMSD (USEPA, 2000b) apply to untransformed reproduction data for a large sample of toxicity tests, including those in which Dunnett's test would not have been used for hypothesis testing. For the purposes of calculating PMSDs, use of Dunnett's test also is acceptable when there is variation among treatments in the number of replicates.

13.13.3.7.13 When the MSD represents a percent reduction from the control that is smaller than the lower PMSD bound for the reproduction endpoint of this test method, 11% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control smaller than 11%. These test concentrations will be considered as not differing significantly from the control for the purpose of determining the NOEC and LOEC.

13.13.3.7.14 When the MSD represents a percent reduction from the control that is larger than the upper PMSD bound for the reproduction endpoint of this test method, 37% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which do not differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control larger than 37%. These test concentrations will be considered as differing significantly from the control for the purpose of determining the NOEC and LOEC.

B. Marine Chronic Method Manual

The following are proposed changes to the marine chronic method manual.

1. Insert the following into Subsection 13.13.1, General, on page 186 of the marine chronic method manual.

13.13.1.4 For hypothesis tests, the percent reduction from the control corresponding to the minimum significant difference (MSD) (see Subsection 9.6.1.4) will be abbreviated herein as PMSD. Upper and lower bounds for the PMSD identified in USEPA (2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described in Subsections 13.13.3.7.13 and 13.13.3.7.14. The upper PMSD bound for the inland silverside growth endpoint is 35% and the lower bound is 12%. The PMSD bounds are intended for use in determining the NOEC and LOEC in multi-concentration tests.

2. Insert the following into Subsection 13.13.3.7, Dunnett's Procedure, on page 210 of the marine chronic method manual.

13.13.3.7.11 Upper and lower bounds for the PMSD (USEPA, 2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described below in Subsections 13.13.3.7.13 and 13.13.3.7.14. The upper PMSD bound is 35% and the lower PMSD bound is 12%.

13.13.3.7.12 To make comparisons with upper and lower bounds for PMSD (USEPA, 2000b), calculate MSD as described in Appendix C, using Dunnett's critical value (d), and calculate PMSD as described in Appendix C, Subsection 1.11.2.2. Calculate these whether or not the Dunnett's test will be used for hypothesis testing. The upper and lower bounds for PMSD (USEPA, 2000b) apply to untransformed growth data for a large sample of toxicity tests, including those in which Dunnett's test would not have been used for hypothesis testing. For the purposes of calculating PMSDs, use of Dunnett's test also is acceptable when there is variation among treatments in the number of replicates.

13.13.3.7.13 When the MSD represents a percent reduction from the control that is smaller than the lower PMSD bound for the growth endpoint of this test method, 12% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control smaller than 12%. These test concentrations will be considered as not differing significantly from the control for the purpose of determining the NOEC and LOEC.

13.13.3.7.14 When the MSD represents a percent reduction from the control that is larger than the upper PMSD bound for the growth endpoint of this test method, 35% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which do not differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control larger than 35%. These test concentrations will be considered as differing significantly from the control for the purpose of determining the NOEC and LOEC.

3. Insert the following into Subsection 14.13.1, General, on page 243 of the marine chronic method manual.

14.13.1.4 For hypothesis tests, the percent reduction from the control corresponding to the minimum significant difference (MSD) (see Sebsection 9.6.1.4) will be abbreviated herein as PMSD. Upper and lower bounds for the PMSD identified in USEPA (2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described in Subsections 14.13.3.7.13 and 14.13.3.7.14. The upper PMSD bound for the mysid growth endpoint is 32% and the lower bound is 12%. The PMSD bounds are intended for use in determining the NOEC and LOEC in multi-concentration tests.

4. Insert the following into Subsection 14.13.3.7, Dunnett's Procedure, on page 269 of the marine chronic method manual.

14.13.3.7.11 Upper and lower bounds for the PMSD (USEPA, 2000b) must be applied to the determination of NOEC and LOEC for the growth endpoint, as described below in Subsections 14.13.3.7.13 and 14.13.3.7.14. The upper PMSD bound is 32% and the lower PMSD bound is 12%.

14.13.3.7.12 To make comparisons with upper and lower bounds for PMSD (USEPA, 2000b), calculate MSD as described in Appendix C, using Dunnett's critical value (d), and calculate PMSD as described in Appendix C, Subsection 1.11.2.2. Calculate these whether or not the Dunnett's test will be used for hypothesis testing. The upper and lower bounds for PMSD (USEPA, 2000b) apply to untransformed growth data for a large sample of toxicity tests, including those in which Dunnett's test would not have been used for hypothesis testing. For the purposes of calculating PMSDs, use of Dunnett's test also is acceptable when there is variation among treatments in the number of replicates.

14.13.3.7.13 When the MSD represents a percent reduction from the control that is smaller than the lower PMSD bound for the growth endpoint of this test method, 12% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control smaller than 12%. These test concentrations will be considered as not differing significantly from the control for the purpose of determining the NOEC and LOEC.

14.13.3.7.14 When the MSD represents a percent reduction from the control that is larger than the upper PMSD bound for the growth endpoint of this test method, 32% (USEPA, 2000b), the following modification must be used when determining the NOEC and LOEC. Identify and document any concentrations which do not differ significantly from the control (using whichever statistical test was selected as appropriate), but which represent a percent reduction from the control larger than 32%. These test concentrations will be considered as differing significantly from the control for the purpose of determining the NOEC and LOEC.