Designation: E2317 – 04 (Reapproved 2012)

Standard Guide for Conducting Renewal Microplate-Based Life-Cycle Toxicity Tests with a Marine Meiobenthic Copepod[1]

This standard is issued under the fixed designation E2317; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the adverse effects of a test material added to seawater, but not to food, on the marine copepod *Amphiascus tenuiremis*, during continuous exposures of individuals, from immediately after birth, until after the beginning of reproduction using a 200 µL renewal microplate-culturing technique. The following data are checked and recorded during the test period: stage-specific survival, number of days it takes for development from a first stage nauplius to a reproductively mature copepod, gender ratios, number of days for a female to extrude first and subsequent broods, number of days between first (and subsequent) brood extrusion(s) and hatching of first-generation nauplii, number of hatched and surviving nauplii, number of unhatched or necrotic eggs and aborted unhatching eggsacs, and the total number of females able to produce viable offspring over the entire mating period. This microplate-based full life-cycle toxicity test has a duration of approximately 17 days for toxicants that do not delay development. These procedures probably will be useful for conducting life-cycle toxicity tests with other species of copepods, although modifications might be necessary.

1.2 These procedures are applicable to most chemicals, either individually, or in formulations, commercial products, or known mixtures, that can be measured accurately at the necessary concentration in water. With appropriate modifications these procedures can be used to conduct tests on temperature, dissolved oxygen, and pH and on such materials as aqueous effluents (see also Guide E1192), sediment pore waters, and surface waters. Renewal microplate tests might not be applicable to materials that have a high oxygen demand, are highly volatile, are rapidly transformed (biologically or chemically) in aqueous solutions, or are removed from test solutions in substantial quantities by the test chambers or organisms during the test. If the concentration of dissolved oxygen falls below 50 % of saturation, or the concentration of test material in the test solution decreases by more than 20 % between renewals, it might be desirable to renew the solutions more often.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory requirements prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1218 Guide for Conducting Static Toxicity Tests with Microalgae

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide.

3.1.1 "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a

[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

Current edition approved Dec. 1, 2012. Published January 2013. Originally approved in 2004. Last previous edition approved in 2004 as E2317– 04. DOI: 10.1520/E2317-04R12.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

different design. "Must" is used only in connection with factors that directly relate to the acceptability of the test (see Section 13).

3.1.2 "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors.

3.1.3 "May" is used to mean "is (are) allowed to," "can," is used to mean "is (are) able to," and "might" is used to mean "could possibly." Therefore, the classic distinction between may and can is preserved, and might is never used as a synonym for either may or can.

3.2 For definitions of other terms in this guide, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to Practice E380.

4. Summary of Guide

4.1 In each of one or more treatments and a control(s), individually isolated *A. tenuiremis* are maintained and reared in 144 or more individual microwell (300 µL total volume) test chambers from immediately after birth (less than 24-h old) until sexual maturity and production of progeny. Microwells (test chambers) are dispersed among at least three replicate 96-well microplates per treatment. Number of treatments, test chambers and organisms per treatment should be based on the purpose of the life-cycle test and the type of data analysis that is to be used to calculate results. Normally, five sublethal treatment concentrations that cause ≤ 10 % acute naupliar mortality may be selected based on an acute 96-h range finding test with 20 nauplii in duplicate microplates over a log-order series of test chemical concentrations spanning zero mortality to 100 % mortality. Beginning with the maximum 10 % lethality concentration, test concentrations normally should decrease by 40 % with each successively lower treatment. In each of one or more control treatments, at least 144 individually isolated copepods are maintained in seawater containing the carrier solvent where appropriate but to which no test material has been added in order to provide *(a)* a measure of the acceptability of the test by giving an indication of the quality of the copepods and the suitability of the seawater, food, test conditions, handling procedures, etc.; and *(b)* the basis for interpreting data obtained from the other treatments. In each of one or more other treatments, at least 144 individual copepods are maintained in seawater to which a selected concentration of test material has been added. Copepod survival and the number of days it takes for development from a first stage nauplius to a reproductively mature copepod are checked and recorded daily. Once copepods have matured to adults, the gender of each copepod is determined, and individual male:female mating pairs are allowed to mate for twelve days in new, isolated microwell test chambers with seawater containing solvent (where appropriate) (control) or test material. The solutions in the microwell test chambers during reproduction are renewed every third day as appropriate under 1.2. Copepods are fed a 1:1:1 mixed algal cell suspension (10^7 cells/mL) every sixth day. Sufficient numbers of algal cells will settle to the microwell bottoms to provide a sufficient six day supply of food even though microwell test solutions are renewed every third day. After single pairing of adult male and female copepods in each control and treatment solution, each mating pair is checked daily for the following: number of days for a female to extrude the first brood, number of days between first (and subsequent) brood extrusion(s) and hatching of first- (and subsequent) brood nauplii, number of hatched and surviving nauplii, number of unhatched or necrotic eggs and aborted eggsacs, and the total number of females able to produce viable offspring over the entire mating period. The test is terminated 12 days past the median time of first brood release in the control treatment(s) to allow for delays in first brood release by copepods exposed to the test material. This microplate-based full life-cycle toxicity test has a duration of approximately 24 days at 25°C. Specified data on the concentration of the test material in test solutions and the survival, growth, gender ratios, and reproduction of each copepod pair are obtained and analyzed to determine the effect(s) of the test material on stage-specific survival, development rates, gender ratios, fertility, and reproduction of the test organisms.

5. Significance and Use

5.1 Protection of a species requires prevention of unacceptable effects on the number, health, and uses of individuals of that species. A life-cycle toxicity test is conducted to determine changes in the numbers of individuals and offspring of a test species resulting from effects of the test material on survival, growth, gender ratios, endocrine function, genetic expression, fertility and reproduction (**1-3**).[3] Information might also be obtained on effects of the material on the health (**4**) and uses of the species.

5.2 Published information about the sensitivities of several meiobenthic copepods to several common metals and organic toxicants have been reviewed (**5**). For most compounds tested/published to date, *A. tenuiremis* is acutely less sensitive than mysid and penaeid shrimp, similarly sensitive as amphipods, and often more sensitive than cladocerans (daphniids, specifically). Reference 96-h aqueous toxicity tests with cadmium at 30 g/kg salinity showed an LC50 for *A. tenuiremis* adults of 213 to 234 µg/L (Chandler, unpub.). Reference toxicant tests with sodium dodecyl sulfate showed a 96-h LC50 of 13.3 to 15.5 mg/L (Chandler,unpubl.). *A. tenuiremis* is a comparatively new toxicity test organism, and an extensive database of species sensitivity to multiple aqueous test compounds is not yet available. Relative to other harpacticoid copepod studies in the literature, *A. tenuiremis* is more chronically sensitive than all other species published to date where there is comparative data (**5**).

5.3 Results of life-cycle tests with *A. tenuiremis* can be used to predict long-term effects at the individual and population levels likely to occur on copepods in field situations as a result of exposure under comparable conditions (**1,2**).

5.4 Results of life-cycle tests with *A. tenuiremis* might be used to compare the chronic sensitivities of different species

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

and the chronic toxicities of different materials, and also study the effects of various environmental factors such as temperature, pH, and ultraviolet light on results of such tests.

5.5 Results of life-cycle tests with *A. tenuiremis* might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E1023) or when deriving water quality criteria for aquatic organisms (**6**).

5.6 Results of a life-cycle test with *A. tenuiremis* might be useful for predicting the results of chronic tests on the same test material with the same species in another water or with another species in the same or a different water. Most such predictions take into account results of acute toxicity tests, and so the usefulness of the results from a life-cycle toxicity test with *A. tenuiremis* is greatly increased by also reporting the results of an acute toxicity test (see Guide E729) conducted under the same environmental conditions.

5.7 Results of life-cycle tests with *A. tenuiremis* might be useful for studying the biological availability of, and structure-activity relationships between, test materials.

5.8 Results of life-cycle tests with *A. tenuiremis* will depend on temperature, quality of food, composition of seawater, condition of test organisms, and other factors.

5.9 Life-cycle tests with *A. tenuiremis* are conducted on copepods reared individually in microwells of 96-well microplates. Thus they can be useful for studying endocrine, pre-zygotic and gender-specific toxicities of test materials (**1-3**).

6. Apparatus

6.1 *Facilities*—Flow-through seawater:sediment brood-stock cultures and static-renewal, but not flow-through, microwell test chambers should be maintained in constant-temperature areas or in incubators. If seawater is not prepared in a batch, it is usually piped directly from the source to an elevated headbox so it can be filtered at 0.45 µm and gravity-fed into recirculating seawater tanks for brood-stock cultures and containers used to prepare test solutions. The headbox should be equipped for temperature control and aeration. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of air through a 0.22-µm bacterial filter might be desirable. The facility should be well ventilated and free of fumes. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, the recirculating seawater tanks for brood-stock cultures should not be in a room where stock solutions or test solutions are prepared, or equipment is cleaned. During testing, organisms should be shielded from disturbances (that is, maintained within a temperature-regulated incubator) to prevent unnecessary stress. White-light fluorescent bulbs (40 W) with light output equal to 3150 lumens per bulb should be used for culturing and testing. A timing device within the incubator should be used to provide a 12-h light and 12-h dark photoperiod. A15- to 30-min 50 % full-intensity light transition period (**7**) should be provided whenever the lights go on or off to reduce the possibility of copepods being stressed by instantaneous changes in light intensity.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any seawater into which copepods will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that adversely affect copepods. In addition, equipment and facilities that contact stock solutions or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and polytetrafluoroethylene (PTFE) plastics should be used whenever possible to minimize dissolution, leaching, and sorption. Stainless steel should not be used for tests on metals. High-density polyethylene plastics may be used for brood-stock tanks and in the seawater supply, but they should be soaked, preferably in flowing seawater, for a week or more before use (**8**). Cast iron pipe should not be used with salt water. Specially designed systems are usually necessary to obtain salt water from a natural water source (see Guide E729). Brass, copper, lead, galvanized metal, and natural rubber should not contact seawater, stock solutions, or test solutions before or during the test. Items made of neoprene rubber or other materials not mentioned previously should not be used unless it has been shown that their use will not adversely affect either survival, growth, sex or reproduction of *A. tenuiremis* (see 13.1.10 and 13.1.11).

6.3 *Test Chambers:*

6.3.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. Through the use of ultra-low attachment polystyrene 96-well microplates, test chambers (that is, experimental units) are physically isolated and test solution cannot flow from one chamber to another. Chambers should be covered and placed in a clean, temperature-regulated incubator to keep out extraneous contaminants and to reduce evaporation of test solution and test material. All chambers in a test must be of identical dimensions and composition.

6.3.2 Ultra-low attachment polystyrene 96-well microplates have a hydrophilic surface coating to minimize sorption of hydrophobic test materials from water. Glass-lined polystyrene microplates have a 250-nm thick glass coating applied uniformly over the microwell walls to serve as yet another type of plastic:seawater barrier. Seawater control copepod survival in both of these microplate types has been > 90 % through 25 days at 25°C ((**1-3**)). The use of all glass microplates has been unsuccessful (greater than 20 % control mortality) and is not recommended for life-cycle toxicity tests with *A. tenuiremis*. Polystyrene microplates require minimal space (a temperature-regulated incubator) for both single exposures and the multi-factorial designs required for toxicant mixture tests. Each microwell chamber within a microplate allows a maximum test solution volume of 300 µL, but a 200 µL test solution volume is desirable to prevent cross-contamination from one microwell to another. Microwells of hydrogel-coated microplates must be hydrated with deionized water for one hour and allowed to air dry prior to the addition of test solutions. Microplates are disposable, and new ultra-low attachment or glass-lined polystyrene microplates should be used for every additional test. Individual treatment and control test chambers (microwells)

should not be combined within any individual microplate to reduce the possibility of cross-contamination.

6.4 *Cleaning*—Glassware used to prepare and store seawater, stock solutions, and test solutions should be cleaned before use. All glassware should be cleaned before each use by washing with laboratory detergent, followed by three distilled or deionized water rinses, 10 % nitric (HNO_3) or hydrochloric (HCl) acid rinse, and at least three distilled or deionized water rinses. Metals, sulfides, and carbonate deposits are removed by the acid rinse. Organic chemicals should be removed by a water-miscible organic solvent (for example, acetone) rinse followed by a distilled or deionized water rinse, or by baking for 8 h at 300 to 400°C. The use of a hypochlorite solution is not recommended, because it is highly toxic to copepods (**5**) and difficult to remove from some materials. At the end of each test, all items that are to be used again should be immediately *(a)* emptied, *(b)* rinsed with tap water, *(c)* cleaned by a procedure appropriate for removing the test material, and *(d)* rinsed at least twice with deionized, distilled, or clean seawater. However, microplates are disposable, and new microplates should be used for all additional tests. Large plastic containers used only for non-toxic seawater may be rinsed after use with distilled or deionized water. They should be used only for toxicity tests and stored in a room that is free from toxic fumes. Glassware and plastics used only for live copepods, not exposed to toxicants, may be cleaned using only distilled, deionized, or clean seawater, since the use of detergents is sometimes detrimental to live copepods.

6.5 *Acceptability*—Before a life-cycle test is conducted in new test facilities, it is desirable to conduct a nontoxicant test in which all test chambers contain clean seawater with no added test material. This test determines *(a)* whether *A. tenuiremis* will survive, grow, and reproduce acceptably in the new facilities, *(b)* whether the food, seawater, or handling procedures are acceptable, *(c)* whether there are any location effects on either survival, growth, gender ratios, fertility or reproduction, and *(d)* the magnitudes of the between-chamber and between-microplate variances.

7. Hazards

7.1 Information on toxicity to humans (**9**), recommended handling procedures (**10**), and chemical and physical properties of the test material should be studied before a test is begun. Many materials can adversely affect humans if precautions are inadequate. Therefore, skin contact with all test materials and solutions should be minimized by wearing appropriate protective gloves (especially when washing equipment or putting hands into test solutions), laboratory coats, aprons, and safety glasses, and by using glass micropipets to remove copepods from test solutions. For all test materials, Materials Safety Data Sheets (MSDS) should be posted and made available to all laboratory personnel who could be exposed knowingly or unknowingly to the test material(s). Special precautions, such as covering microwell test chambers (that is, with a microplate lid) and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Special procedures might be necessary with radiolabeled materials (**11**) and with test materials that are, or are suspected of being, carcinogenic (**12**).

7.2 Although this life-cycle toxicity test generates little hazardous waste (less than 3 L) and the disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

7.3 Cleaning of equipment with a volatile solvent, such as acetone, should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

7.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

7.6 Use of ground fault systems and leak detectors is strongly recommended to help prevent electrical shocks because salt water is a good conductor of electricity.

8. Toxicity Test Water

8.1 *Requirements*—The seawater should *(a)* be in adequate supply, *(b)* be acceptable to *A. tenuiremis*, *(c)* be of uniform quality, and *(d)* except as stated in 8.1.4, not unnecessarily affect results of the test.

8.1.1 The seawater should allow satisfactory survival, growth, and reproduction of *A. tenuiremis* (see 13.1.10 and 13.1.11).

8.1.2 The quality of the seawater should be uniform during the test. During the test each measured salinity should be 30 g/kg, and the difference between the highest and the lowest measured salinities should be less than 3 g/kg. Each measured pH should be between 8.0 and 8.3.

8.1.3 The seawater should not unnecessarily affect results of a life-cycle test with *A. tenuiremis* because of such things as sorption or complexation of test material. Therefore, except as stated in 8.1.4, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L.

8.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of a life-cycle test with *A. tenuiremis*, it will be necessary to use water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be available or be conducted in a more usual seawater to facilitate interpretation of the results in the special water.

8.2 *Source:*

8.2.1 *Natural Salt Water*—If natural salt water is used, it should be obtained from an uncontaminated area known to support a healthy, reproducing population of *A. tenuiremis* or a comparable sensitive species. The water intake should be

positioned to minimize fluctuations in quality and the possibility of contamination, and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. A specially designed system might be necessary to obtain salt water from a natural water source. To ensure uniform quality, water should be monitored as in 8.4. These precautions are intended to ensure that test organisms are not apparently stressed by water quality during holding, acclimation, and testing and that water quality does not unnecessarily affect test results. The water should meet the criteria given in 8.1.

8.2.2 *Artificial Salt Water*—Artificial salt water can be prepared by adding commercially available sea salt or specified amounts (see Guide E729) or reagent-grade chemicals **(13)** to high-quality water with *(a)* conductivity less than 1 µS/cm and *(b)* either TOC less than 2 mg/L or chemical oxygen demand (COD) less than 5 mg/L. Acceptable water can usually be prepared using properly operated deionization or distillation units. Artificial salt water should be intensively aerated before use, and aging for one to two weeks might be desirable. If a residue or precipitate is present, the solution should be filtered before use. The water should meet the criteria given in 8.1.

8.2.3 Chlorinated water must never be used in the preparation of water for toxicity tests, because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals **(14)**. Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Municipal drinking water is not recommended for use because in addition to residual chlorine, it often contains unacceptably high concentrations of metals, and quality is often highly variable (see Guide E729).

8.3 *Preparation:*

8.3.1 Seawater used in the life-cycle toxicity test should be aerated intensively by using air stones, surface aerators, or column aerators **(15)** before addition of test material. Adequate aeration will bring the pH and concentration of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be greater than 90 % of saturation **(16)** to help ensure that dissolved oxygen concentrations in the test chambers are acceptable **(17)**.

8.3.2 Seawater used in the life-cycle toxicity test should be passed through a filter effective to 5 µm or less to remove suspended particles and organisms from the water. Seawater that might be contaminated with facultative pathogens should be passed through a properly maintained ultraviolet sterilizer **(18)** or a filter effective to 0.45 µm or lower.

8.3.3 If necessary, the salinity should be reduced by diluting the seawater with high-quality deionized or distilled water. Salinity can be raised by addition of clean filtered oceanic water or artificial sea salts. It should be shown that the salt causes no adverse effects on survival, growth, gender ratios or reproduction of *A. tenuiremis* at the concentration used.

8.3.4 Fresh seawater used in the test should be prepared within two days of the test and stored in covered containers at room temperature. Seawater should be aerated to ≥ 90 % oxygen saturation and filtered at ≤ 0.45 µm prior to addition of water to test chambers. Longer holding periods (that is, greater than two days) may result in lower control survival. It is recommended to age artificial seawater for one to two weeks before use. Artificial seawater may be aged up to one month prior to testing. Sufficient starting or renewal water should be prepared at one time for all test chambers.

8.4 *Characterization:*

8.4.1 The following items should be measured at least twice each year and more often if such measurements have not been made semi-annually for at least two years or if natural seawater is used: salinity (or chlorinity), pH, particulate matter, TOC, organophosphorous pesticides, organic chlorine (or organochlorine pesticides and polychlorinated biphenyls (PCBs)), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc.

8.4.2 The methods used (see 12.3) should be either *(a)* accurate and precise enough to adequately characterize the seawater or *(b)* have detection limits below concentrations that have been shown to adversely affect the survival, growth, or reproduction of *A. tenuiremis*.

9. Test Material

9.1 *General*—The test material should be reagent grade or better, unless a test on formulation, commercial product, or technical-grade or use-grade material is specifically needed. Before a test is begun, the following should be known about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities. For example, impurities constituting more than about 1 % of the material.

9.1.2 Solubility and stability in seawater.

9.1.3 Measured acute toxicity to *A. tenuiremis*.

9.1.4 Precision and bias of the analytical method at the planned concentration(s) of test material.

9.1.5 Estimate of toxicity to humans.

9.1.6 Recommended handling procedures (see 7.1).

9.2 *Stock Solution:*

9.2.1 In some cases the test material can be added directly to seawater and dissolved. If the material is non-polar and poorly soluble in seawater, it may be dissolved in a solvent carrier to form a stock solution that is then added to seawater. If a stock solution is used, the concentration and stability of the test material in it should be determined before beginning the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.2 Except for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is seawater, although filtration or sterilization, or both, of the water before introduction of test material might be necessary. If the hardness of the seawater will not be affected, deionized water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(19)**. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material,

such as chloride or sulfate salts of organic amines, sodium, or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of necessary minimum amounts of strong acids and bases.

9.2.3 If a solvent other than seawater is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect either survival, growth, or reproduction of *A. tenuiremis*. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals (**20**), low volatility, and high ability to dissolve many organic chemicals. Other water-miscible organic solvents such as dimethylformamide, methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms, and acetone is very volatile. If an organic solvent is used, it should be reagent grade or better and its concentration in any test solution should not exceed 0.1 mL/L. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient in a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution.)

9.2.4 If a solvent other than water is used, *(a)* at least one solvent control, using solvent from the same batch used to make the stock solution, should be included in the test, and *(b)* a seawater control should be included in the test. If no solvent other than water is used, a seawater control should be included in the test.

9.2.4.1 If the concentration of solvent is the same in all test solutions that contain test material, the solvent control should contain the same concentration of solvent.

9.2.4.2 As this copepod life-cycle test incorporates chronic endpoints that may not follow a classic logistic dose-response curve, use of a single highest-dose "worst case" solvent concentration control may not provide sufficient information about potential low-dose solvent effects. Therefore, if the concentration of solvent is not the same in all test solutions that contain test material, either *(a)* a solvent test should be conducted to determine whether either survival, growth, or reproduction of *A. tenuiremis* is related to the concentration of solvent over the range used in the toxicity test or *(b)* such a solvent test should have already been conducted using the same seawater and *A. tenuiremis*. If either survival, growth, or reproduction is found to be related to the concentration of solvent, a life-cycle test with *A. tenuiremis* in that seawater is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival, growth, nor reproduction is found to be related to the concentration of solvent, a life-cycle toxicity test with *A. tenuiremis* in that same seawater may contain solvent concentrations within the tested range, but the solvent control should contain the highest concentration of solvent present in any of the other treatments.

9.2.4.3 If the test contains both a seawater control and a solvent control, the survival, growth, and reproduction of *A. tenuiremis* in the two controls should be compared. If a statistically significant difference in either survival, growth, or reproduction is detected between the two controls, only the solvent control may be used for meeting the requirements of 13.1.10 and 13.1.11 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be pooled and used for meeting the requirements of 13.1.10 and 13.1.11 and as the basis for calculation of results.

9.2.5 If a solvent other than seawater is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvent on the results of the test.

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to provide a good estimate of the highest concentration of test material that will unacceptably affect neither survival, growth, nor reproduction of *A. tenuiremis*, the test concentrations (see 11.1.1.1) should bracket the best prediction of that concentration. Such a prediction is usually based on the results of a static acute toxicity test (see Guide E729) on the test material using the seawater and less than 24-h old nauplii. Acute tests should be conducted with and without food added to the seawater because food used in the life-cycle test sometimes affects the results of the acute test. If an acute to chronic ratio has been determined for the test material with a species of comparable sensitivity, the results of the acute test with *A. tenuiremis* can be divided by the acute to chronic ratio. If no other useful information is available, the highest concentration of test material in a life-cycle test with *A. tenuiremis* is often selected to be equal to the lowest concentration that caused adverse effects in a comparable acute test.

9.3.2 In some (usually regulatory) situations, it is necessary only to determine whether one specific concentration of test material reduces survival, growth, or reproduction. For example, the specific concentration might be occurring in surface water, resulting from direct application of the material to a body of water, or the solubility limit of the material in water. When there is interest only in a specific concentration, it is often necessary only to test that concentration (see 11.1.1.2).

10. Test Organisms

10.1 *Species—A. tenuiremis* has been used extensively for acute and life-cycle toxicity tests for the past ten years (**21-23**). *A. tenuiremis* (Mielke, 1974) is a meiobenthic harpacticoid copepod in the family Diosaccidae that is easily cultured in sediments or seawater in the laboratory. In the field, it dwells in oxidized muddy sediments of intertidal to subtidal habitats of the Atlantic and Baltic Seas (**24**). The major life-stages of *A. tenuiremis* are shown in Fig. 2. In microplates at 25°C and 30 g/kg, *A. tenuiremis* passes through six larval naupliar stages in six to seven days, and five copepodite stages in six to seven days. Females are sexually mature after the fifth and last copepodite molt (that is, the twelfth life stage). They then mate and produce their first clutch in one to two days. Sperm transfer is via a membranous spermatophore sac attached externally by the male to the female genital pore. Nauplii hatch within two to three days of egg extrusion and reach the first copepodite stage in six to seven days. See Appendix X1 for detailed information on utility of *A. tenuiremis* for toxicity tests. *A. tenuiremis* may