



Standard Guide for Conducting Renewal Microplate-Based Life-Cycle Toxicity Tests with a Marine Meiobenthic Copepod¹

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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 E 1192), 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

- E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)
- E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians
- E 943 Terminology Relating to Biological Effects and Environmental Fate
- E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
- E 1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids
- E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians
- E 1218 Guide for Conducting Static 96-h Toxicity Tests with Microalgae
- E 1847 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

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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.

specified condition, unless the purpose of the test requires a 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 E 729, Terminology E 943, and Guide E 1023. For an explanation of units and symbols, refer to Practice E 380.

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 $\leq 10\%$ 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 microw-

ell 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 (5). Reference toxicant tests with sodium dodecyl sulfate showed a 96-h LC50 of 13.3 to 15.5 mg/L (5). *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 and the chronic toxicities of different materials, and also study

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

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 E 1023) 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 E 729) 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, prezygotic 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. A 15- to 30-min 50 % full-intensity light transition period (11) 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 (12). 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 E 729). 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 20°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 (HNO₃) 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 (7), recommended handling procedures (8), 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 (9) and with test materials that are, or are suspected of being, carcinogenic (10).

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 E 729) or reagent-grade chemicals (13) to high-quality water with (a) conductivity less than 1 $\mu\text{S}/\text{cm}$ 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 E 729).

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 $\geq 90\%$ oxygen saturation and filtered at $\leq 0.45\ \mu\text{m}$ 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 orga-

nochlorine 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 E 729) 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 be obtained from Dr. G. Thomas Chandler, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA 29208.

10.2 *Age*—Life-cycle tests with *A. tenuiremis* should be started with individuals less than 24-h post release from the egg sac.

10.3 *Source*—Copepods can be purchased from G. T. Chandler at the University of South Carolina. All copepods in a test should be from the same brood stock. The two previous generations should have been raised from birth using the same food, seawater, and temperature as used in the life-cycle test.

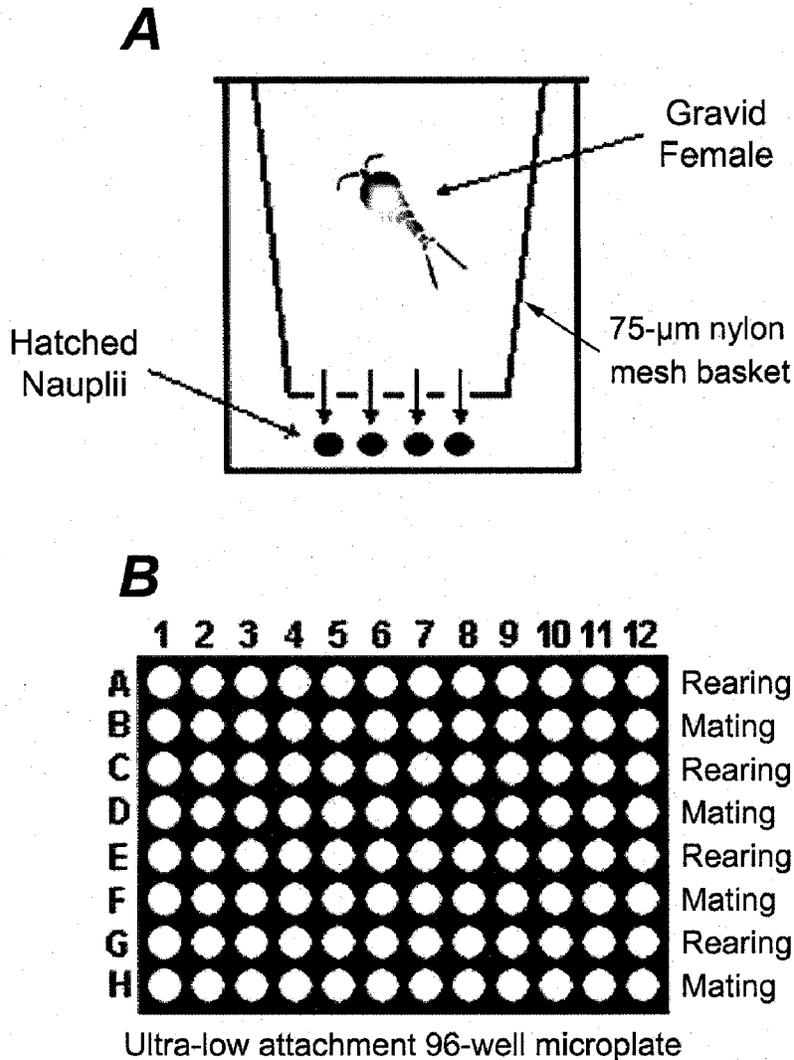


FIG. 1 Schematic of (A) Naupliar Collection Apparatus, and (B) Microplate Test Layout and Design

This will not only acclimate the copepods, but will also demonstrate the acceptability of the food, seawater, and handling procedures before the test is begun.

10.4 Brood-Stock:

10.4.1 Brood-stock may be obtained from a wild population from an unpolluted area, but it is recommended that cultured copepods be used for life-cycle testing.

10.4.2 *A. tenuiremis* has been successfully cultured in sediments (25) and is controlled by (a) a toxicant-free muddy sediment substrate consisting of clay, silt, fine-sand particles less than 0.125 mm in size, (b) a 5- μ m filtered carbon-polished seawater source saturated with oxygen and relatively free of ammonia (less than 30 μ g-NH₄/L), and (c) a varied microalgal diet (see Appendix X2). Successful cultures may be started with as few as 30 gravid copepods, but can reach maximum density more quickly if 100 or more gravids are introduced initially. Cultures should be fed twice per week to support adequate reproduction. One hundred gravid copepods will often yield a peak density culture of approximately 50 copep-

ods \cdot cm⁻³ sediment in two to three months if food, sediment, and water quality are high.

10.5 Food—At least twice per week, copepod stock sediment cultures should be fed a 1:1:1 mixed algal cell suspension, in excess, of a chlorophyte (for example, *Dunaliella tertiolecta*), a chrysophyte (for example, *Isochrysis galbana*), and a diatom (for example, *Phaeodactylum tricorutum*) alga. In microplate test chambers, each copepod should be fed 2 μ L of a 1:1:1 mixed algal cell suspension (10⁷ cells/mL) of a chlorophyte, chrysophyte and diatom (for example, *Dunaliella tertiolecta*, *Isochrysis galbana*, and *Phaeodactylum tricorutum*) every six days. At this recommended rate, food is not limiting for individual copepod survival, development, and reproduction, even though microwell test-material solutions are renewed every three days in this lifecycle test. Sufficient numbers of algal cells will settle to the microwell bottoms to provide a sufficient six day supply of food. Copepods should not be fed more than the recommended rate as this will cause

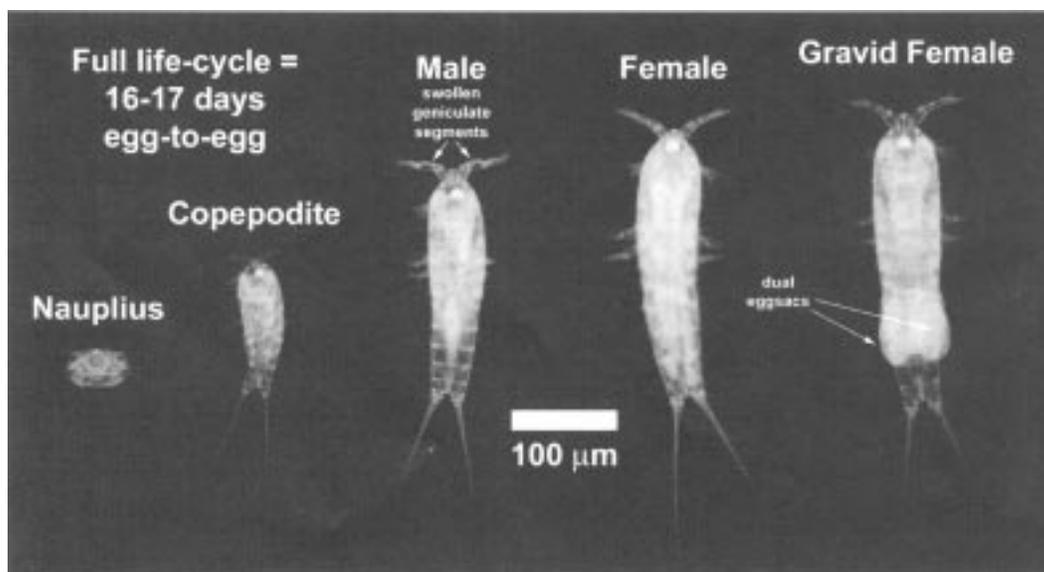


FIG. 2 Photomicrographs of *Amphiascus tenuiremis* Major Life Stages

excessive accumulation of algal cells in microwells and lead to reduced dissolved oxygen.

10.6 *Handling*—*A. tenuiremis* should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that the copepods are not unnecessarily stressed, and gravid females will not lose their externally carried egg sacs. When collecting gravid *A. tenuiremis* females, monoculture stock sediments should be sieved gently over a 125 μm stainless steel sieve, with the retained sediment fraction and copepods washed into a sterile, plastic petri dish (10 cm diameter) using seawater at the same salinity as used for the life-cycle test. High-quality borosilicate glass pasteur pipets 146 mm in length should be used for collection and transfer of gravid female *A. tenuiremis* under a dissection stereomicroscope and cool fiber-optic illumination. Copepods should be introduced into solutions beneath the air to water interface. Copepods that touch dry surfaces or are dropped or injured should be discarded.

10.7 *Harvesting Young*—Less than 24-h old young are obtained by first collecting gravid females as stated in 10.6, and then dividing gravid females equally among wells of a six-well, polystyrene plate filled individually with 15 mL of 0.2- μm filtered seawater. Within each well, a 75- μm nylon mesh basket is suspended to retain females approximately 3 mm above the well bottom. The basket allows newly hatched non-swimming nauplii to fall through the mesh and away from adult females (Fig. 1). After 24 h, the mesh basket and post-hatch females are removed, and nauplii are collected from all wells and transferred to sterile, plastic petri dishes using silanized (see 11.4.3) fine-tipped (0.1 mm diameter) borosilicate glass pasteur pipets.

10.8 *Quality*—To increase the chances of an acceptable test (see 13.1), the test should not begin with nauplii that were from moribund or dying (that is, immobile) copepods. All nauplii are handled and checked using light microscopy. All 24-h old nauplii must be alive before beginning the test. Nauplii are non-swimming so their survivability may be checked under a

stereomicroscope by gently tapping on the petri dish holding the representative nauplii. Representative copepods from the brood stock should be analyzed for the test material if it might be present in the environment.

11. Procedure

11.1 *Experimental Design:*

11.1.1 Decisions concerning aspects of experimental design, such as the dilution factor, number of treatments, and numbers of test chambers and pairs of copepods per treatment, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 14). One of the following two types of experimental designs will probably be appropriate in most cases.

11.1.1.1 A life-cycle test intended to allow calculation of an endpoint usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the seawater or solvent control(s), or both, (see 9.2.3) copepods are exposed to seawater where no test material has been added. Except for the control(s) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.6, five properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of chronic toxicity is particularly nebulous (see 9.3.1), six or seven concentrations might be desirable.

11.1.1.2 If it is necessary to determine only whether a specific concentration reduces survival, growth, or reproduction (see 9.3.2), then tests of only that concentration and the control(s) are necessary. Two additional concentrations at about one-half and two times the specific concentration of concern are desirable to increase confidence in the results.

11.1.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the

experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned (26). Because test solution can not flow from one test chamber to another (see 6.3.1), the test chamber is the experimental unit. However in this multichamber microplate approach, test chambers are nested within plates and are not strictly independent. Thus, it is recommended that test chambers (microwells) for a given treatment or control be distributed over at least three microplates to meet statistical assumptions of sample independence, and to avoid concerns of pseudoreplication (27). As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom increases, and, therefore, the width of the confidence interval on a point estimate decreases and the power of a significance test increases. With respect to factors that might affect results within test chambers and, therefore, the results of the test, all chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in four rows per microplate. Treatments should be randomly assigned to individual microplates, but no more than one treatment or control per microplate. A completely randomized nested design (for example, microwells within microplate within treatment or control) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of test chambers per treatment should be calculated from (a) the expected variance within test chambers, (b) the expected variance between test chambers within a treatment, and (c) either the maximum acceptable confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing (26). If such calculations are not made, at least three 96-well microplates (that is, 48 individual test chambers per 96-well microplate) and 144 physically isolated individual copepods should be used for each treatment. This sample size maximizes the number of microwells over three replicate microplates. Replicate test chambers and microplates are necessary in order to allow estimation of experimental error (26). If each test concentration is more than 60 % of the next higher one and if the data are to be analyzed using regression analysis, fewer copepods per concentration of test material, but not the control treatment(s), may be used. Because of the importance of controls in the calculation of results, it might be desirable to use more copepods for the control treatment(s) than for each of the other treatments; however a fully balanced design usually is most informative.

11.2 *Dissolved Oxygen*—The seawater should be aerated to ≥ 90 % oxygen saturation and filtered at ≤ 0.45 μm prior to the addition of test material at each 3-day test solution renewal. The concentration of dissolved oxygen in each test chamber solution should be from 60 to 100 % of saturation (22) over each 3 day renewal period during the test. Passive aeration in the control and test solutions should be the same throughout the test in all test chambers, including the control(s). Direct aeration of the test chambers is not possible due to the minimal allowable volume (200 μL) of seawater per test chamber, and should be avoided because it might stress copepods, resuspend

fecal matter, and greatly increase volatilization of the test material. The small individual test chamber volumes may not allow direct measurement of all physico-chemical and test solution values every 3 days. In those instances, individual microwell volumes may be removed by pipette and pooled within microplates within treatments to yield sufficient volumes for measurement where necessary.

11.3 *Temperature:*

11.3.1 Life-cycle tests with *A. tenuiremis* should be conducted at 25°C. Other temperatures may be used to study the effect of temperature on *A. tenuiremis* or to study the effect of temperature on the chronic toxicity of the material to *A. tenuiremis*.

11.3.2 The internal temperature of the incubator should be checked every three days to ensure that the digital or analog temperature display on the exterior of the incubator is accurate. For the incubator in which air temperature is measured, the time-weighted average measured air temperature at the end of the test should be within 1°C of the selected temperature. The difference between the highest and lowest time-weighted air temperature averages for the incubator should not be greater than 2°C.

11.4 *Beginning the Test:*

11.4.1 Recommended conditions for conducting a renewal microplate-based life-cycle toxicity test with *A. tenuiremis* are outlined in Table 1. A general activity schedule is outlined in Table 2. Ultra-low attachment polystyrene 96-well microplates are hydrated with deionized water for one hour and allowed to air dry prior to use. Three microplates each with 48 microwells (that is, a total of 144 microwells) should be used for each treatment at test initiation. Each microplate is set up by utilizing a total of four rows (12 microwells per row) for rearing of juvenile copepods and four rows (12 microwells per row) for pairing and mating of adult copepods. This design allows for rearing of juvenile copepods and mating of adult copepods to take place in one microplate (Fig. 1). By alternating rows for rearing and mating, full utilization of the plate is obtained and fewer microplates are needed. This design can be manipulated depending on the sample size necessary for the experiment.

11.4.2 To ensure that all treatments receive the same seawater, the batch of seawater should be large enough to fill all test chambers for all test concentrations and the control(s) and to perform chemical analyses. Seawater should be spiked with the test material from stock solutions using an analytical grade glass or PTFE capillary (± 0.2 μL) pipette. All test solutions should be covered and mixed using a magnetic stir bar and stir plate for one hour prior to addition to test chambers. The concentration of test material in a fresh test solution should be no more than 50 % higher or lower than the nominal concentration. If the difference is more than 50 %, the cause should be identified. If the concentration in the test chamber is too high, the stock solution or test solution might have been incorrectly prepared. If the concentration is too low, possible additional causes are microbial degradation, hydrolysis, oxidation, reduction, photolysis, sorption, and volatilization. If the copepods

TABLE 1 Test Conditions for Conducting a Renewal Microplate-Based Life-Cycle Toxicity Test with *Amphiascus tenuiremis*

Parameter	Conditions
1. Test type:	Aqueous life-cycle toxicity test with renewal of overlying seawater.
2. Temperature:	25 ± 1°C
3. Light quality:	Wide-spectrum fluorescent light
4. Light output:	3150 lumens per bulb
5. Photoperiod:	12L:12D
6. Test chamber:	300-µL hydrophilic microwell
7. Overlying seawater volume:	200 µL
8. Renewal of overlying seawater:	1 volume exchange every 3 d
9. Age of organisms:	Less than 24-h old nauplii
10. Number of organisms/chamber:	1 per chamber until adult, then 1 male:female pair/chamber
11. Number of replicate chambers/treatment:	Depends on the objective of the test. 144 microwells over 3 replicate microplates are recommended for routine testing (see 11.1.3).
12. Feeding:	Each well receives 2 µL of a fresh, 10 ⁷ cells·mL ⁻¹ , 1:1:1 mixed algal cell suspension every 6 d (see 10.5).
13. Seawater aeration:	Greater than 90 % saturation prior to the addition of test material. Direct aeration of the test chamber is not possible (see 11.2).
14. Overlying seawater:	Natural or artificial seawater (30 g/kg) passed through a filter effective to 5 µm or less.
15. Overlying water quality:	Salinity, pH, and dissolved oxygen in initial and renewal control solutions prior to the addition to test chambers.
16. Test duration:	Approximately 24 d
17. Endpoints:	Survival; number of days it takes for development from a first stage nauplius to a reproductively mature copepod; number of days for a female to extrude the first brood; number of days between first brood extrusion and hatching of first-generation nauplii; number of hatched and surviving nauplii; number of unhatched or necrotic eggs; number of aborted non-hatching eggsacs; total number of surviving females able to produce viable offspring over the entire mating period (for example, % fertility).
18. Test acceptability:	Minimum mean control survival of ≥80 % between test initiation and male:female pairing; minimum mean control survival of ≥90 % between male:female pairing and test end. At least 70 % of the paired first-generation females in the control(s) produce >4 hatched offspring. See Table 3 for additional performance-based criteria.

TABLE 2 General Activity Schedule for Conducting a Renewal Microplate-Based Life-Cycle Toxicity Test with *Amphiascus tenuiremis*

Day	Activity
-1	Sieve out gravid females from stock sediment culture and divide gravid females equally among wells of a six-well, polystyrene plate filled individually with 15 mL of 0.2-µm filtered seawater. Within each well, suspend a 75-µm nylon mesh basket to retain females approximately 3 mm above the well bottom. Over a 24-h period, allow newly-hatched non-swimming nauplii to fall through the mesh basket away from adult females (Fig. 1).
0	Remove the mesh basket and post-hatch females and collect nauplii from all well bottoms and transfer to sterile, plastic petri dishes using silanized (see 11.4.3) fine-tipped (0.1 mm diameter) borosilicate glass pasteur pipets. Hydrate ultra-low attachment polystyrene 96-well microplates with deionized water for 1 h and allow to air dry prior to use. Transfer 1 nauplii into each microwell test chamber by releasing organisms under the surface of the water. Remove overlying transfer seawater so that approximately 5 µL of seawater remains. Add 200 µL control or test solution, and 2 µL mixed algal cell suspension to each test chamber. Cover and place microplates in a temperature-regulated incubator set at 25°C with a 12-h light and 12-h dark photoperiod.
1 to 24	Renew control and test solutions every 3 d. Record water quality parameters and test material concentration in all test solutions at each renewal. Feed each developing copepod a mixed algal cell suspension every 6 d. Check and record the following data daily: individual survival and the number of days it takes each individual to develop from a first stage nauplius to a first stage copepodite to a reproductively-mature adult copepod. At maturity, record number of males and females per treatment and control.
12-15	Randomly pair control and treatment adult males and females into new test chambers (that is, one male:female pair per test chamber). Remove overlying transfer seawater so that approximately 5 µL of seawater remains. Add 200 µL control or test solution, and 2 µL mixed algal cell suspension to each test chamber. Cover and place microplates in a temperature-regulated incubator set at 25°C with a 12-h light and 12-h dark photoperiod. Note: If the test material delays naupliar and/or copepodite development time to the adult stage, >12 days may be required before sufficient numbers of adult males and females are available for mating. Virgin controls may be held unmated for at least 2 weeks to allow treatment copepods sufficient time to reach maturity.
12 to 24	Check and record the following data daily: number of days for a female to extrude the 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. End the test by discarding test solution (generally less than 1 L) and disposing microplates into appropriate waste containers.

are being exposed to substantial concentrations of one or more degradation products, measurement of the product(s) is desirable (see 11.9.1).

11.4.3 After less than 24-h old nauplii have been collected according to 10.7, single nauplii are randomly selected and gently transferred into designated microwells using glass pipettes treated with an air-dried solution of 80, 3, and 1.5 % ethyl alcohol, isopropyl alcohol, and ethyl sulfate, respectively. This silanization solution can be purchased from VWR Scientific Products, and should be stored at 4°C in darkness. This

solution coats the inside of the glass pipette with a clear polymer that allows easier transfer of *A. tenuiremis* nauplii. This silanization of glass pipettes is not acutely toxic to *A. tenuiremis*.

11.4.4 After all wells are successfully loaded with a single nauplius, the overlying transfer seawater is removed under a stereomicroscope using an analytical grade 500-µL glass syringe so that approximately 5 µL of seawater remains. This standardizes the starting volume in each well and allows for minimal dilution of the test solutions.

11.4.5 Once the overlying water is removed, 200 μL of control(s) or test solution is added, aspirated, and added again to the appropriate wells using a graduated, glass capillary pipette. After test solutions have been added, each well should receive 2 μL of a fresh, 10^7 cells·mL⁻¹ 1:1:1 mixed algal cell suspension as described in 10.5. Each microplate is covered and placed in a temperature-regulated incubator set at 25°C with a 12-h light and 12-h dark photoperiod.

11.5 *Renewing Test Solutions*—Every three days, test solutions should be aspirated from each microwell test chamber using an analytical grade 500- μL glass syringe so that approximately 20 μL of test solution remains. Care should be taken to ensure that copepods are not aspirated into the syringe. If copepods are aspirated, the corresponding test chamber must be disqualified from the test. Fresh test solution should be prepared and added to the appropriate test chambers as described in 11.4.2 and 11.4.5. Feeding should occur regularly at least once every six days (see 11.4.5).

11.6 *Pairing*—After 11 to 13 days at 25°C, most control copepodites should have molted into sexually mature adults. Sexes are dimorphic with females and males reaching 0.40 mm and 0.25 to 0.30 mm in length, respectively (Fig. 2). Males are streamlined in shape and have a swollen geniculate segment on their first antenna to clasp the female. Treatment copepodites may exhibit delayed development and maturation depending on the toxicity of the test material(s). Using a glass pipette, sexually mature males and females are removed from each control and treatment test chamber and placed briefly into separate same-sex 50-mL glass crystallizing dishes (one per microplate) containing fresh control or treatment solution, respectively. Adult copepods from the same microplate are placed in the same treatment solution as in the test chambers to ensure a continuous test solution exposure. One single male and female copepod are then randomly removed from the appropriate crystallizing dishes and placed into a previously unused microplate well (Fig. 1) in their original microplate. This is performed for the control(s) and test concentration(s) until all available males and females are successfully formed into mating pairs. The maximum number of mating pairs that can be evaluated per treatment and control (for example, 72 pairs/treatment with even gender ratios and 100 % survival/maturation to adult) depends upon the initial number of nauplii per treatment and control, and the final number and ratios of adult virgin females and males surviving to maturity in each treatment and control at the time of mating (for example, at 11 to 13 days for seawater controls at 25°C). Test chambers are next loaded with 200 μL of fresh control or test solution, and each mating pair is fed 2 μL of fresh algal mixture prepared as described in 11.4.5. The effect of the test material on survival, growth, and reproduction cannot be determined accurately if any non-treatment factor that affects one or more of these endpoints is too dissimilar between experimental units. Because survival, growth, and reproduction might be affected by the number of parental and first-generation copepods in the chamber and the concentration or amount of available food, the best experimental design is to remove hatched young at least every third day (that is, during the time when seawater is renewed). Maximizing the number of test chambers (that is,

nauplii) per treatment and control will increase the number of mating pairs per treatment and control, and improve the power of the experimental design. Statistically, the best use of any specific number of copepod mating pairs is to place each male:female pair alone in a separate test chamber to maximize statistical independence.

11.7 *Duration of Test*—A complete life-cycle test would end when the last parental generation copepod died. However, shorter tests will probably provide adequate data on the effect of the test material on the survival, growth, and reproduction of *A. tenuiremis*. A test with *A. tenuiremis* at 25°C should not be terminated before 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. The test should be extended, however, if previously unaffected copepods in any treatment are adversely affected near the intended end of the test.

11.8 *Biological Data:*

11.8.1 Survival should be checked daily in each test chamber using inverted stereomicroscopy. The criteria for death of copepods are opaque white coloration, immobility, and lack of reaction to gentle tapping of the microplate. The gender of dead parental generation copepods (if mature) should be recorded. In each test chamber, live copepods should be counted at the beginning of the test, at the time of pairing, and daily after pairing. Missing copepods should be recorded as such and not recorded as dead.

11.8.2 The number of days it takes for development from a first stage nauplius to a reproductively mature copepod should be checked and recorded daily using inverted stereomicroscopy. Copepod gender should be checked and recorded at reproductive maturity.

11.8.3 After single pairing of adult male and female copepods in each control and treatment solution, each mating pair should be checked daily using inverted stereomicroscopy. The following data are recorded daily: number of days for a female to extrude the first brood, number of days between first brood extrusion and hatching of first-brood nauplii, number of days to subsequent brood extrusions and hatch, number of hatched and surviving nauplii per brood and test period, 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.

11.8.4 Morphological and histological examination of parental copepods alive at the end of the test in each treatment might be desirable. Biochemical and histological examination and measurement of the test material in exposed copepods will probably not be possible unless additional copepods are exposed specifically for such purposes.

11.8.5 All copepods used in a test should be preserved in 4 % borate buffered formalin or glutaraldehyde for future reference, or be destroyed at the end of the test.

11.9 *Other Measurements:*

11.9.1 *Water Quality*—Salinity, pH, and dissolved oxygen should be measured in the initial and renewal control solutions prior to the addition to test chambers. Weekly determinations of particulate matter, TOC, and total dissolved gases in fresh renewal test solutions are desirable. If test materials are

expected to exhibit rapid breakdown or cause high oxygen demand, it might be desirable to collect and pool individual microwell volumes within microplates within each treatment at the end of each 3 day renewal period for dissolved oxygen, pH and test material measurements. Alternatively, additional microplate replicates might be set up for the sole purpose of collecting physico-chemical measurements on microwell solutions through time.

11.9.2 *Temperature*—Since microwells restrict direct test-solution temperature measurement throughout the test, the incubator air temperature should be (a) monitored daily by inspecting the digital display on the exterior of the temperature-regulated incubator and (b) measured and recorded every three days in the interior of the incubator.

11.9.3 *Test Material*:

11.9.3.1 The concentration of test material in each treatment should be measured frequently enough to establish its average and variability. If the test material is an undefined mixture, such as a leachate or complex effluent, direct measurement is probably not possible nor practical. Concentrations of such test materials will probably have to be monitored by such indirect means as turbidity or by measurement of one or more components.

11.9.3.2 The concentration of test material should be measured from each fresh renewal control and treatment solution prior to the addition to test chambers. The concentration of test material from old test solutions from individual microwells probably cannot be measured due to the limited amount of test solution volume (approximately 200 μL) available for analysis, but see 11.9.1.

12. Analytical Methodology

12.1 The methods used to analyze test material water samples might determine the usefulness of the test results because all results are based on measured concentrations. For example, if the analytical method measures any impurities,

reaction, or degradation products along with the parent test material, results can be calculated only for the whole group of materials and not for parent material by itself, unless it is demonstrated that such impurities and products are not present.

12.2 If samples of seawater, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately (28) to minimize loss of test material by microbial degradation, hydrolysis, oxidation, reduction, photolysis, sorption, and volatilization.

12.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements where ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources (29).

12.4 Methods used to analyze test food (see 10.5) or test copepods (see 10.8) should be obtained from appropriate sources (30).

12.5 The precision and bias of each analytical method used should be determined in an appropriate matrix, that is, in water samples from a brood-stock tank or fresh test solution, in food, and in copepods. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

13. Acceptability of Test

13.1 Specific requirements for test acceptability are detailed in Table 3. A life-cycle test with *A. tenuiremis* usually should be considered unacceptable if one or more of the Table 3 requirements are violated, except that if, for example, temperature was measured numerous times, a deviation of more than 3°C (see 13.1.15) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature had been measured more often.

TABLE 3 Test Acceptability Requirements for a Renewal Microplate-Based Life-Cycle Toxicity Test with *Amphiascus tenuiremis*

1.	All microwell test chambers were not identical.
2.	Treatments were not randomly assigned to test chamber microplates.
3.	A required seawater control or solvent control was not included in the test or, if the concentration of solvent was not the same in all treatments that contained test material, the concentration of solvent in the range used affected survival, growth, or reproduction of <i>A. tenuiremis</i> .
4.	The test was started with copepods from more than one brood stock or with copepods from a brood stock that had not been maintained for at least two generations using the same food, water, sediments, and temperature as used in the test.
5.	The test was started with copepods more than 24-h old.
6.	Individual copepods were not impartially assigned to separate test chambers.
7.	There was less than 200 μL of solution per parental copepod in the microwell test chamber when the solutions were renewed every three days.
8.	The test was terminated before the minimum duration specified in 11.7.
9.	More than 20 % of the parental control copepods died between test initiation and adult pairing.
10.	More than 10 % of the parental control copepods died 3 days past the median time of first brood release in the control treatment(s) .
11.	More than 30 % of the paired first-generation females in the control(s) failed to produce young, or they produced <4 offspring per female on average.
12.	Salinity, dissolved oxygen, temperature, and concentration of test material were not measured as specified in 11.9.
13.	The highest and lowest measured test salinities differed by more than 3 g/kg.
14.	The time-weighted average measured dissolved oxygen at the end of the test for any pooled microplate treatment was not from 60 to 100 % of saturation.
15.	The difference between the time-weighted average measured air temperatures above any two microplates was more than 2°C.
16.	Any individual measured air temperature value above a microplate was more than 3°C different from the mean of the time-weighted average measured air temperature for the individual microplates.
17.	At any one time, the difference between the measured air temperatures above any two microplates was more than 2°C.
18.	The measured concentration of the test material in any treatment was less than 30 % of the time-weighted average measured concentration for more than 10 % of the duration of the test.
19.	The measured concentration of test material in any treatment was more than 30 % lower or higher than the time-weighted average measured concentration for more than 5 % of the duration of the test.
20.	An assessment should be made of the significance of test material in the seawater, in the control treatment(s), in the food (see 10.5), and in the brood stock (see 10.8).

14. Calculation of Results

14.1 The primary data to be analyzed from a life-cycle test with *A. tenuiremis* are those on (a) stage-specific survival of developing copepods, (b) development times from nauplius-to-copepodite and copepodite-to-adult, (c) gender ratios of surviving adults, (d) the percentage of virgin females able to release at least one brood after being paired with same-treatment virgin males, (e) the number of necrotic eggs and aborted eggsacs produced per female, (f) the number of successfully hatched young from the first and any subsequent broods in the test period, and (g) the concentration of test material in the test solutions of each treatment at each test solution renewal.

14.2 The variety of procedures that can be used to calculate results of life-cycle toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (a) the advantages and disadvantages of various alternative procedures and (b) appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of the results should be appropriate to the experimental design (see 11.1). The major alternative procedures and points that should be considered when selecting and using procedures for calculating results of a life-cycle toxicity test with *A. tenuiremis* are discussed in Appendix X3 and Guide E 1191.

14.3 First-generation copepods are counted from pairing day until the end of the test. Missing animals are excluded from counts.

14.4 It may be desirable to analyze time required from mating to first brood release for each pair.

15. Report

15.1 The record of the results of an acceptable life-cycle test with *A. tenuiremis* should include the following information either directly or by reference to available documents.

15.1.1 Names of test and investigator(s), name and location of laboratory, and dates of initiation and termination of test.

15.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

15.1.3 Source of the seawater, its chemical characteristics, and a description of any pretreatment.

15.1.4 Source of the brood stock, scientific name, name of person who identified the organisms and the taxonomic key used, acclimation and culture procedures used, and age of the copepods at the beginning of the test.

15.1.5 Description of the experimental design, microplate test chambers and covers, the volume of solution in the chambers, number of first-generation copepods, test chambers, and chambers per treatment, lighting, and renewal schedule.

15.1.6 Procedure used to prepare food, concentrations of test material and other contaminants in the food, and feeding method, frequency, and ration.

15.1.7 Range and time-weighted average measured concentration of dissolved oxygen (as percent of saturation) and description of any aeration performed on test solutions before or during the test.

15.1.8 Range and time-weighted average of measured test air temperature and the method(s) of measuring or monitoring, or both.

15.1.9 Schedule for obtaining samples of test solutions and the methods used to obtain, prepare, and store the samples.

15.1.10 Methods used for, the results (with standard deviations or confidence limits) of chemical analyses of water quality and concentration(s) of test material (in fresh solutions), impurities, and reaction and degradation products, including validation studies and reagent blanks.

15.1.11 A table of data in sufficient detail to allow independent statistical analyses on survival, growth, sex and reproduction of copepods in each test chamber in each treatment, including the control(s).

15.1.12 Methods used and results of statistical analyses of the data.

15.1.13 Summary of general observations on other effects.

15.1.14 Results of all associated acute toxicity tests.

15.1.15 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

15.2 Published reports should contain enough information to clearly identify the procedures used and the quality of the results.

APPENDIXES

(Nonmandatory Information)

X1. AMPHIASCUS TENUIREMIS

X1.1 *Ecology and Morphology*—*A. tenuiremis* c.f. Mielke is an easily-cultured, diosaccid harpacticoid copepod that is amphiatlantic in distribution ranging from the North Sea/Baltic intertidal to the southern Gulf of Mexico (24). *A. tenuiremis* is an epifaunal to deep burrowing (0 to 15 mm) species that ingests sediments for diatoms and bacteria, and produces burrowing larvae with no swimming ability. Sexes are dimorphic with females reaching 0.4 mm in length and

males 0.25 to 0.30 mm. Males are also streamlined in shape and have a swollen geniculate segment on their first antenna to clasp the female.

X1.2 *Growth and Reproduction*—*A. tenuiremis* has a generation time of 14 to 16 days at 25°C. Females are sexually mature after the fifth copepodite stage (that is, in the twelfth life stage). They then mate and produce their first clutch in two

to three days. Isolated virgin females can be fertilized successfully up to 14 days post adulthood, and females will not extrude viable eggs unless paired with a reproductively-functional mature male. Females will produce eight to nine

clutches over their median lifetime of 49 ± 2 days. Nauplii hatch within two to three days of egg extrusion and reach the copepodite stage in six to seven days.

X2. STOCK CULTURE

X2.1 Sediment Culturing Procedures:

X2.1.1 *General Information*—Successful copepod culture is keyed by three important conditions: (1) a toxicant-free muddy sediment substrate consisting of clay, silt, fine sand particles less than 0.100 mm in size (median grain diameter approximately 0.004 to 0.02 mm); (2) a highly-filtered carbon-polished seawater source saturated with oxygen and relatively free of ammonia (less than $30 \mu\text{g-NH}_4/\text{L}$; pH = 8.0 to 8.3); (3) a varied diet of at least three easily cultured phytoplankton species (for example, a chlorophyte, *Dunaliella tertiolecta*, a chrysophyte, *Isochrysis galbana*, and a diatom, *Phaeodactylum tricorutum*). All marine algae are cultured in nutrient-enriched seawater (see Guide E 1218) and can be purchased from the University of Texas Culture Collection.

X2.1.2 *Preparation of Clean Stock Sediments*—Collect toxicant-free muddy sediments from a pristine estuarine habitat (for example, North Inlet, SC) and pass sediments through 0.5-mm and 0.1-mm sieves into deionized water. Resuspend and wash three times after settling, and then centrifuge (3 min at 4000 RCF) to remove 50 to 60 % of the water. Homogenize “dry” sediment pellets in an electric blender and place into 1-L Griffin beakers, cover loosely, and autoclave for 15 min at 125°C and 30 psi. Store autoclaved sediment stocks sealed at 4°C until use. Prepare culture sediment substrate by taking 100 g autoclaved stock, blending it for 3 min at high speed with 300-mL 5- μm -filtered seawater, and then pouring the blended stock through a 0.063-mm sieve into 3.5 L of filtered seawater in a 4-L Griffin beaker. Allow sediments to settle for 30 min, and then aspirate away the supernatant water. Resuspend the

remaining settled sediments again in 3.5 L filtered seawater and allow to resediment for 24 h at 4°C. After 24 h, aspirate away all supernatant water, leaving approximately 300 mL wet sediments.

X2.1.3 *Addition of Copepods*—Pour wet sediments (from X2.1.2) into 1-L crystallizing dishes filled with 500 mL seawater and allow to settle for 1 h. Inoculate the sediments with the copepod taxon of interest. Typically greater than 100 gravid copepods are required to start a viable culture. Wet sediment media has a consistent water-to-solids percentage of 87:13 to 88:12 using this technique. Sediment organic carbon content (based on C:H:N analysis) consistently ranges from 3.8 to 4.2 % among North Inlet, SC, USA sediment lots after passing them through these procedures. Raw North Inlet field sediments range from 4 to 6 % organic carbon by C:H:N.

X2.1.4 *Culture Maintenance and Feeding*—Flush each copepod culture dish slowly (approximately 3 to 5 mL/min) but continuously with polished natural or synthetic seawater from a recirculating or ambient 0.005-mm filtered seawater system. Feed cultures a 1:1:1 mixed algal cell suspension in slight excess of what they consume (for example, approximately $2.5 \cdot 10^8$ centrifuged cells for a mature culture) twice per week. Culture algal cells to exponential growth using standard f/2 enriched seawater media (31) and centrifuge for 12 min at 4700 RCF. Remove the supernatant, resuspend algae in clean sediment, microwave for 15 s (700 W) to destroy swimming abilities, cool to 4°C, and pipet into each culture. One hundred gravid copepods will often yield a peak density culture in 2 to 3 months if food, sediment, and water quality are high.

TABLE X2.1 Nutrient Stock Solutions for Marine Algal Culture (31)

	gm/L
Nutrients	
NaNO ₃	75.0
NaSiO ₃	30.0
NaPO ₄	5.0
Trace Metals	
MnCl ₂	180.0
ZnSO ₄	22.0
CoCl ₂	10.0
CuSO ₄	9.8
Na ₂ MoO ₄	6.3
Vitamins	
Thiamine	0.200
Vitamin B ₁₂	0.001
Biotin	0.00104

NOTE—Prepare each nutrient stock solution separately. Prepare each stock solution with filtered deionized water and store at 4°C. To prepare 1 L working solution in seawater, add 1 mL of each nutrient stock solution and 1 mL trace metals stock solution to 1 L artificial seawater and autoclave for 25 min. After bottles have cooled completely, add 0.5 mL vitamin stock solution.

X3. STATISTICAL GUIDANCE

X3.1 *Introduction*—The goals of statistical analysis are to summarize, display, quantify, and provide objective yardsticks for assessing the structure, relations, and anomalies in data. The data display and statistical techniques most commonly used to achieve these goals are (a) preliminary and diagnostic graphical displays, (b) pairwise comparison techniques such as *t*-tests and 2 by 2 contingency table tests, (c) analysis of variance (ANOVA) and corresponding contingency table tests,

(d) multiple comparison techniques for simultaneous pairwise comparison of other treatment groups with control groups, (e) concentration-effect curve analyses, and (f) multiple regression. If used correctly, each of these techniques can provide useful information about the results of an acceptable life-cycle toxicity test with *A. tenuiremis*. For specific guidance regarding statistical analysis of life-cycle toxicity data, refer to Guide E 1191 or E 1847.

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