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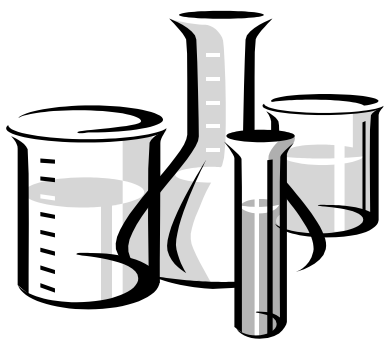
Ecological Effects Test Guidelines

OCSPP 850.1735: Spiked Whole Sediment 10-Day Toxicity Test, Freshwater Invertebrates



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to http://www.epa.gov/ocspp and select "Test Methods & Guidelines" on the navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.1735: Spiked whole sediment 10-day toxicity test, freshwater invertebrates

(a) **Scope**

(1) **Applicability.** This guideline is intended for use in meeting testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, *et seq.*). It describes procedures that, if followed, would result in data that would generally be of scientific merit for the purposes described in paragraph (b) of this guideline.

(2) **Background.** The source materials used in developing this harmonized OCSPP test guideline are ASTM E 1706, Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates (see paragraph (j)(1) of this guideline); and U.S. EPA Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates (EPA 600/R-99-064), (see paragraph (j)(12) of this guideline). Other source materials are listed in paragraph (j) of this guideline. Mention of trade names or commercial products in this guideline does not constitute endorsement or recommendation for use.

(b) **Purpose.** This guideline is intended for use in determining the toxicity to benthic invertebrates from sub-chronic exposure to sediment concentrations of chemical substances and mixtures subject to environmental effects test regulations. This guideline prescribes a test in which benthic freshwater macroinvertebrates are exposed to test substances incorporated in sediment. Naturally-derived clean sediment or formulated sediment is spiked with different concentrations of test substance. The test results are used to determine relationships between the test substance in sediment and a biological response such as mortality and growth in benthic invertebrates. The Environmental Protection Agency will use data from this test in assessing the hazards and risks a test substance may present in the aquatic benthic environment.

(c) **Definitions.** The definitions in OCSPP 850.1000 apply to this test guideline. In addition, the following more specific definitions apply to this guideline:

Clean refers to a sediment or water that does not contain organisms or concentrations of constituents such as metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, ammonia levels, *etc*. which cause apparent stress to the test organisms or reduce their survival or growth.

Immobilization refers to the lack of movement when gently prodded.

Interstitial water or *pore water* is water occupying the space between sediment or soil particles.

Overlying water is the water placed over sediment in a test vessel during a test.

Sediment refers to deposits of organic matter, sand, soil, and other particulate matter which form a substrate in an aquatic system. In this guideline, the definition also includes

formulated or artificially-prepared particulate material that is intended to form a substrate below overlying water in a test vessel.

Sediment, bulk (or whole) is the solid-phase sediment plus its associated pore water which has undergone minimal manipulation. The term bulk sediment is used synonymously with whole sediment.

Sediment concentration is the ratio of the weight of test substance(s) to the dry weight of bulk (or whole) sediment.

Sediment concentration, acid-volatile sulfide normalized refers to the normalization of the sediment concentration to the acid-volatile sulfide content of the sediment and is the ratio of the weight of test substance to the weight of acid-volatile sulfide in the whole sediment sample.

Sediment concentration, organic-carbon normalized refers to the normalization of the sediment concentration to the organic-carbon content of the sediment and is the ratio of the weight of test substance to the weight of organic-carbon in the whole sediment sample.

Spiked sediment refers to natural or artificial sediment to which a test substance has been added and incorporated for experimental or study purposes.

(d) **General considerations**

(1) **Summary of the test.** Whole sediment toxicity test procedures are outlined for the amphipod, *Hyalella azteca*, and the midge, *Chironomus dilutus*. The duration of sub-chronic whole sediment tests is 10 days using spiked sediment as well as an appropriate control(s). Test organisms are monitored during the test for sediment avoidance and other toxic effects observable without disturbing the sediment. At test termination, survival and growth are determined. The results of the test can be expressed as the 10-day median lethal concentration (10-d LC_{50}), the 10-day median growth effect concentration (10-d EC_{50}), or the 10-day no observed effect concentration (10-d NOEC) and 10-day lowest observed effect concentration (10-d LOEC) for survival and growth. For pesticides, the NOEC and LOEC are the preferred 10-d endpoints. For industrial chemicals, the LC_{50} and/or EC_{50} are the preferred 10-d endpoints.

(2) **General test guidance.** The general guidance in OCSPP 850.1000 applies to this guideline except as specifically noted herein.

(3) **Range-finding test.** A range-finding test is usually conducted to establish the appropriate test substance sediment concentrations to be used for the definitive test. In the range-finding test, the test organisms are generally exposed to a series of widely-spaced concentrations of the test substance (*e.g.*, 1, 10, 100 milligrams per kilogram (mg/kg) dry weight). The details of the range-finding test do not have to be the same as for definitive testing in that the number of replicates, the number of test organisms, and duration of exposure may be less than that used in definitive testing, and nominal sediment concentrations of the test substance are acceptable. In addition the types of observations

made on test organisms may not be as detailed or as frequently observed as that of a definitive test. It may be possible that "water-only" range-finding tests are sufficient to establish the concentrations for definitive testing, based upon predicted pore-water concentrations.

(4) **Definitive test.** The goal of the definitive test for pesticides is to determine the NOEC and LOEC values for the most sensitive measure of effect, either mortality or growth. The selected test concentrations should bracket the NOEC and LOEC values.

The test may also be designed to determine the concentration-response curves for mortality and growth effects, the 10-d LC_{50} for mortality and the 10-d EC_{50} for growth along with their respective 95% confidence intervals, the slopes of the concentration-response curves, their associated standard errors, and 95% confidence intervals. The selected test concentrations should bracket the expected 10-d LC_{50} for mortality and 10-d EC_{50} for growth, or whichever is more sensitive.

Either test design should use at least five sediment concentrations of the test substance, plus appropriate control(s). Test concentrations are typically expressed as mg/kg dry weight for sediment and mg/L for interstitial (pore) water. Depending on the test substance, endpoints may be normalized to organic carbon content or acid-volatile sulfide content of the sediment and to dissolved pore water concentrations. Analytical confirmation of test concentrations in bulk sediment and interstitial (pore) water should be performed. Summaries of the test conditions for a test with the amphipod *H. azteca* and with the midge *C. dilutus* are presented in Table 1 and Table 2 of this guideline, respectively. The test validity elements for a test with the amphipod *H. azteca* and with the midge *C. dilutus* are listed in Table 3 and Table 4, respectively.

(5) **Limit test.** In some situations, it is only necessary to ascertain that the 10-d NOEC or 10-d LC_{50}/EC_{50} for survival and growth is above a certain limit concentration (*i.e.*, 10-d NOEC or 10-d LC_{50}/EC_{50}>limit concentration). In a limit test, at least eight replicate test vessels with 10 test organisms per replicate are exposed to a single "limit concentration," with the same number of replicates and test organisms in appropriate control(s). For pesticides, 100 milligrams of active ingredient per kilogram of sediment (mg a.i./kg) (dry weight), when estimated environmental concentrations are not expected to exceed 100 mg a.i./kg, may be used as the limit concentration. For most industrial chemicals, 100 milligrams per kilogram of sediment (mg/kg) (dry weight) is considered appropriate as the limit concentration. These sediment limit concentrations should be appropriately adjusted upwards if use, production, disposal, or other releases result in sediment concentrations above these limits. Except for the number of test concentrations, an acceptable limit test follows the same test procedures, is the same duration, and has the same number of controls as the multiple-concentration definitive test (see Table 1 and Table 2 of this guideline). Limit tests, like definitive tests, include analytical confirmation of the limit concentration in bulk sediment and the interstitial water concentration. For pesticides, if there is a statistically significant reduction in mortality or growth at the limit concentration as compared to the control(s) (*i.e.*, no observed effect concentration (NOEC) less than (<) limit concentration), a definitive test should be conducted. For industrial chemicals, if the effect level for mortality or inhibition of growth at the limit

concentration compared to the control(s) is 50% or greater, then a definitive test should be conducted.

(e) **Test standards**

(1) **Test substance.** The test substance should be reagent grade (or technical grade for pesticides) unless the test is designed for a specific formulation, mixture, or degradate. For pesticides, if more than one active ingredient constitutes a technical product, the technical grade of each active ingredient should be tested separately, in addition to the combination, if applicable. OCSPP 850.1000 lists the type of information that should be known about the test substance before testing. The test substance is spiked into the sediment as described in OCSPP 850.1000 (f)(2)(i).

(2) **Test duration.** The duration of the sub-chronic test with freshwater amphipods or midges is 10 days.

(3) **Test organisms.**

(i) **Species**. The recommended test species are the amphipod, *Hyalella azteca,* and the midge, *Chironomus dilutus* (formally *C. tentans*). These species have contact with the sediment, tolerate varying sediment physical and chemical characteristics, are easily cultured in the laboratory, have a database demonstrating relative sensitivity to various chemicals, and have been used in inter-laboratory studies (paragraph (j)(12)). *H. azteca* are epibenthic detritivores that burrow into the sediment and are found in both freshwater and saline waters up to 29 parts per thousand (ppt). The larval portion of the *C. dilutus* life cycle is spent in a tunnel or case within the upper layers of benthic sediments of lakes, rivers, and estuaries. The feeding habits include both filter feeding and ingesting sediment particles. Chironomids often constitute a significant portion of the benthic biomass. Life history characteristics of *H. azteca* and *C. dilutus* are found in references in paragraphs (j)(8) and (j)(12) of this guideline. The submitter should consult with the Agency prior to using other benthic invertebrate species in testing.

The test organism used should be identified using an appropriate taxonomic key. Since *C. tentans* is now known as *C. dilutus* in North America, it first needs to be verified that the chironomid is actually *C. dilutus*, and the reference in paragraph (j)(8) can be used to help identify the morphological differences between *C. tentans* and *C. dilutus*.

All organisms should be as uniform as possible in age and size. For amphipods, the test is initiated with 7- to 10-d old organisms. In addition for a given test, a narrow range in size and age of organisms (*e.g.*, 24-h range in age) is necessary to reduce potential variability in growth at the end of the test. Measurements of length should be made on at least 20 organisms or measurements of weight on at least 80 organisms just prior to test initiation.

For midges, second- to third-instar chironomids, 10 days old, are used to start the test, with at least 50% of the organisms at the third instar. For a given test, a narrow range in size and age of organisms (*e.g.*, 1-d range in age) is necessary to reduce potential variability in growth at the end of the test. Larvae should develop to the third instar within 9 to 11 days after hatching at a temperature of 23 degrees Celsius (°C). The instar stage of midges may be confirmed on a subsample of the population used to initiate the test based on head capsule width or weight and length of midges at the beginning of a test. Average head capsule width has been documented as 0.20 millimeters (mm) for the second instar and 0.38 mm for the third instar (paragraph (j)(12) of this guideline). Measurements of length, weight, and head capsule width should be made on at least 20 individuals prior to test initiation. Average length of midge larvae at test initiation should be 4-6 mm, while average dry weight should be 0.08-0.23 mg per individual (paragraph (j)(12) of this guideline).

All organisms in a test should be from the same source. Organisms may be obtained from laboratory cultures or from culture facilities (see paragraph (j)(12) of this guideline). Obtaining organisms from wild populations should be avoided unless organisms are cultured through several generations in the laboratory and the sensitivity of the wild population can be documented. If organisms are shipped, temperature and dissolved oxygen should be measured upon arrival to determine if the organisms might have been unacceptably stressed. Details on procedures for obtaining known-age amphipods are found in paragraphs (j)(1) and (j)(12) of this guideline.

(ii) **Holding and acclimation.** The same water used for the culture should be used for testing. *H. azteca* and *C. dilutus* can be cultured in a variety of waters (see OCSPP 850.1000), using either static or static renewal procedures. In static systems, periodic renewal of overlying water is recommended. Cultures should be maintained at 23 °C and a photoperiod should be selected from regimes of 12 hours light:12 hours dark to 16 hours light:8 hours dark at an luminance of about 100 to 1080 lux (approximately 10 to 100 foot-candles (ft-c)). A 15 to 30 minute transition period between light and dark is suggested. Lighting should be constant and continuous throughout the holding and acclimation. A variety of substrates (cotton gauze or maple leaves and artificial substrates for amphipods; sand or shredded paper toweling for midges) have been used for culturing. Details and discussion of acceptable culture procedures for both species are found in paragraphs (j)(1) and (j)(12) of this guideline.

During the acclimation period, mortalities should be recorded, and the following recommendations should be applied:

(A) Mortalities of greater than 10% of the population during acclimation: rejection of entire batch;

(B) Mortalities of between 5 and 10% of the population during acclimation: acclimation continued for additional 7 days;

(C) Mortalities of less than 5% of the population during acclimation: acceptance of batch.

(iii) **Health status and condition.** A group of organisms should not be used for a test:

(A) If more than 5% of the culture dies or shows signs of stress during the 2 days preceding the test;

(B) If they have been used in a previous test, either in a treatment or in a control group.

(iv) **Care and handling.** Test organisms should be handled as little as possible but when necessary it should be done as carefully and quickly as possible.

Test organisms should be introduced into the overlying water below the air-water interface. A wide-bore pipette or a smooth glass tube has been used in transferring organisms. *H. azteca* should be held and fed under the same conditions as the mass culture for at least 48 hours prior to test initiation to help eliminate animals injured during handling.

(v) **Diet and feeding.** Feeding during testing is necessary to prevent starvation; however, overfeeding should be avoided. If excess food collects on the sediment, a fungal or bacterial growth may develop, in which case feeding should be suspended for one or more days. A drop in the dissolved oxygen below 2.5 mg/L during a test may indicate that the food added is not being consumed. Feeding may need to be suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in all treatments. Records of feeding rates and the appearance of the sediment surface each day should be maintained.

(A) **Amphipods.** Extensive testing using the 42-d test method for *H. azteca* showed that the previously recommended diet of 1 mL yeast, cerophyl®, and trout chow (YCT) per day limited growth compared to alternative diets. Through this work, two new diets were developed, one based on YCT supplemented with fish food flakes, and a second using a suspension of commercially-available marine diatoms (*Thalassiosira weissflogii*) supplemented with fish food flakes. Details on dietary recommendations for *H. azteca* are from paragraph (j)(13) of this guideline.

During the test, food is added to each exposure chamber on days 0 to 9. The ration fed is increased for days 7 to 9 of the exposure; specific rates are provided in Table 1. To avoid flushing food out of the exposure chambers, feeding should take place soon after completion of a water renewal, allowing the food time to settle to the sediment surface before the next water renewal. The diets are added as solids suspended in water, and care is needed to deliver a consistent amount of food to each exposure

chamber. The food containers should be agitated to insure a homogeneous suspension every time food solution is withdrawn.

During culturing, feeding amphipods the same diet as used during the test is recommended. Feeding rates should be determined by the laboratory based on the size of the culture.

(1) **Selection of diet to be used**. Either diet, YCT+flake food or diatoms+flake food are acceptable for use in this method, though only one should be used for the duration of any single test, and it is recommended that laboratories use one or the other consistently to maximize consistency in test results over time. Comparisons of these two diets using the *H. azteca* 42-d method have not shown consistently superior performance for either diet over the other, which is why both are included here. A possible weakness of the YCT diet is that the lack of specificity in the component ingredients and the use of an open-air microbial incubation might introduce variability among batches of YCT, though a clear demonstration of negative consequences resulting from this possibility is not known. The YCT+flake food diet appears to result in slightly lower dissolved oxygen concentrations in overlying water compared to the diatoms+flake food diet, though the additional dissolved oxygen suppression by the YCT+flake food diet is usually small (≤1 mg/L).

(2) **Preparation of flake food suspension**. The flake food solution is prepared from flake food (e.g., TetraMin® Tropical Flakes (Tetra Holding (US), Inc., Blacksburg, VA) or functional equivalent). The dry flakes are crushed through a U.S. standard (#50) sieve (300-µm opening). The sieved flakes are apportioned by weight and added to a measured amount of deionized water. The concentration of fine flakes can be adjusted so that the proper ration is delivered in a consistent volume of 1 mL by multiplying the target daily ration by the volume of food prepared (in mL). For example, 100 mL a food solution delivering 0.25 mg/mL can be prepared by adding 0.25 mg/mL * 100 mL = 25 mg flakes to 100 mL of deionized water. The flake food solution is gently agitated to wet and distribute the flakes (not blended) and stored under refrigeration; fresh solution should be prepared every 2 to 3 days.

(3) **Preparation of diatom suspension**.

(a) **Diatom source.** The diatom, *T. weissflogii*, is a unicellular microalgae found in marine, brackish, and freshwater environments (Kipp *et al*., 2013), for which a stabilized concentrate of *T. weissflogii* can be used (*e.g*., Instant Algae® TW1200 (Reed Mariculture, Inc, Campbell,

CA) or functional equivalent). Some laboratories have had success using diatom suspensions prepared from laboratory cultures of *Mayamaea* sp., and *Nitzschia* sp. (D.J. Soucek, Illinois Natural History Survey, Champaign, IL, unpublished data); substitution of other species of diatoms should be shown to support comparable performance of *H. azteca* before being used in tests.

(b) **Preparation of diatom food solution.** If the *T. weissflogii* concentrate is in a saline solution, then washing is needed to reduce the salt content; this can be accomplished by adding fresh water to aliquots of concentrate in a 4:1 ratio, mixing, then centrifuging to re-concentrate the diatoms. This process may need to be repeated, after which conductivity is used to verify that ion concentrations have been reduced. The specifics of this process can be adjusted to the equipment available in a particular laboratory; the following steps provide one example using 100-mL centrifuge tubes.

1. Combine about 20 mL of diatom concentrate and about 80 mL of clean fresh (control) water in each of eight 100-mL centrifuge tubes and mix well. Adding water using a squirt bottle may help suspend the diatoms which may stick to the side of the tube.

2. After mixing, centrifuge the tubs for about 15 min at about 1700 x g (other centrifugation durations and intensities may be suitable also). Decant the supernatant and the add water to the same total volume used initially, mix well, and repeat the centrifugation process.

3. After the second centrifugation, decant the supernatant and transfer the concentrated diatoms to a single container and add water to bring the diatom solution to about three times the original volume of diatoms that were rinsed and mix (in this example 20 mL concentration x 8 tubes x 3 = 480 mL).

4. Check the conductivity of the final suspension; it is typically about 700 µS/cm; if it is substantially higher than that, another centrifugation cycle can be used to reduce conductivity further.

5. Store the washed concentrate under refrigeration; the shelf life after washing is typically two weeks.

(c) **Adjusting solids content and calculating ration volumes**. The solids content of the washed diatom solution is adjusted to a consistent value to allow delivery of known rations. The selection of solids content and delivery volumes is arbitrary, though the example described results in delivery volumes that are easily delivered with most laboratory pipettes; other concentrations and volumes may be used as long as they deliver the appropriate ration. First, the solids concentration of the washed diatoms solution is determined by placing measured volumes (*e.g.*, 3 mL) into triplicate tared weigh pans, drying for 24 h at 70 °C, cooling the pans, then determining the mass of dry solids. Based on this value, water is as needed to the washed diatom solution to bring it to 5 mg solids/mL. This solution can then be used to deliver 0.5 mg in 100 µL, 0.75 mg in 150 µL, 1 mg in 200 µL, 1.5 mg in 300 µL, 2 mg in 400 µL, and 2.5 mg in 500 µL.

(4) **Preparation of YCT**. The preparation procedure for YCT solution is described in Appendix B of USEPA (2000), (see paragraph (j)(12) of this guideline); YCT solution may also be purchased from commercial suppliers. The solids content of YCT solution should be adjusted to about 1800 mg total solids/L to provide a consistent ration.

(B) **Midge.** Midges are fed a fish flake food suspension (*e.g.*, Tetrafin®). Midges are fed 1.5 mL of fish flake food suspension (at 4.0 g solids/L) in each test vessel daily during the test. During culturing, for about 600 larvae in 6 to 8 liters of culture water, fish flake food suspension has been used at a final concentration of about 0.04 mg dry solids per mL of culture water. A stock suspension of the solids is prepared in culture water such that a total volume of 5 mL of food suspension is added daily to each culture vessel.

Recent research suggests that growth of the midge can be improved by providing flaked fish food as the suspension of fine fish food flakes instead of the blended form (D.R. Mount, USEPA, Duluth, MN; unpublished data). In addition, providing food as fine flakes also allowed growth that was actually greater than that from the previous standard diet of 6 mg/d of blended suspension using less food. After extensive experiments using different rations and different rates of increasing ration, a diet consisting of a pre-test (day-1) addition of 6 mg, 2 mg/d for test days 0 to 3, 4 mg/d for days 4 to 6, and 6 mg/d for days 7 to 9 appeared to be the best for balancing the competing demands of promoting midge growth while minimizing depression of dissolved oxygen from excess

food (D.R. Mount, USEPA Duluth, MN and C.G. Ingersoll, USGS, Columbia, MO; unpublished data).

(4) **Administration of test substance**. For tests with pesticides, the routine test medium is spiked formulated sediment (see paragraph (c)(4) of OCSPP 850.1000). For testing of industrial chemicals, the test substance is either spiked into naturally-derived sediment or formulated sediment depending upon the purposes of the study. If naturally-derived sediment is used, it should be fully characterized and free of organisms that might compete with or consume the test organisms.

(i) **Preparation of spiked sediment.** Spiked sediment of a given concentration is prepared by: 1) adding the test substance directly to the sediment as an aqueous solution for soluble test substances; 2) adding the test substance directly to the sediment in a dry form for a water insoluble solid; or 3) by sorbing the test substance to sand and then mixing the treated sand into the sediment for water insoluble test substances. Stock solutions of test substance in organic solvents should not be added to the sediment mixture because they can affect the concentration of dissolved organic carbon in pore water. See OCSPP 850.1000 paragraph (f)(2)(i) for guidance on preparation of spiked sediment.

If a vehicle is used, a solvent sediment control should be included in the test in addition to the negative sediment control. The selected vehicle should not affect the test organisms at the concentration used or interfere with test results. The solvent sediment control should be prepared from the same batch of solvent and should contain the same concentration of vehicle used in the test treatments (preferably) or the highest concentration of vehicle used in any test treatment. See (e)(5) of this guideline for additional details on negative and solvent controls.

(ii) **Placement of sediment in test vessels.** Test sediment (unspiked control sediment and spiked sediment at various test concentrations and aged as described in OCSPP 850.1000 paragraph (f)(2)(i)(C)) should be thoroughly mixed and added to test vessels the day before (day -1) the start of the test. The degree of homogeneity should be inspected visually. Homogeneity may be quantified by taking replicate subsamples and analyzing for total organic carbon (TOC), test substance sediment concentration, and particle size.

Equal amounts of sediment should be added to each test vessel on the basis of volume or weight. To minimize disturbance of sediment, overlying water should be poured gently along the sides of the test vessels or poured over a turbulence reducer (*e.g.*, a disk cut from polyethylene, nylon, or Teflon, or a glass Petri dish attached to a glass pipette) positioned above the sediment. Turbulence reducers should be rinsed with overlying water between replicates, and individual turbulence reducers used between treatments. This test is typically conducted with 300 mL test vessels which contain 100 mL of sediment and 175 mL of overlying water. Tests have also successfully been run using 100 mL beakers containing 30 mL of sediment and 60 mL of water. The renewal of overlying water should

commence on day -1. The test begins once organisms are added to the test vessels (day 0).

(iii) **Sediment test concentrations.** At least five sediment test concentrations are used for definitive testing, plus the appropriate control(s). A range-finding test can be used to establish the appropriate sediment test concentrations for the definitive test (see paragraph (d)(3) of this guideline). For scientifically sound estimates of a given point estimate on the dose-response curve (*e.g.*, LC_{50}, EC_{50}) test substance concentrations should immediately bracket the point estimates(s) of concern. For a hypothesis-based endpoint study, the lowest test treatment level should be at or below the NOEC for the most sensitive effect measure (survival or growth). OCSPP 850.1000 provides guidance on selection of test concentrations. For a limit test, there is a single sediment treatment concentration, plus the appropriate control(s). Guidance on the limit sediment concentration is provided in paragraph (d)(5) of this guideline.

(5) **Controls**

(i) Every test includes a negative sediment control, and a solvent sediment control is also included if a vehicle is used. Sediment controls are created using the same sediment, overlying water, and animal test populations and undergo the same manipulations and procedures as the test substance treatment groups, except that no test substance is added. Sediment controls are kept under the same environmental conditions as test substance treatment groups. Control sediment is essentially free of contaminants and is used routinely to assess the acceptability of a test. Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination.

(ii) A test is not acceptable if:

(A) If mean survival in either the negative or solvent sediment control is less than 80% of exposed amphipods, or less than 70% of exposed midges at the end of the test;

(B) If growth of 2.5x in either the negative or solvent sediment control does not occur by the end of the test for amphipods or if the average size in either the negative or solvent sediment control is not at least 0.48 mg ash-free dry weight (AFDW) at the end of the test for midges.

(iii) For pesticides, if a solvent is used, both a negative and solvent control are recommended. If a non-volatile solvent is used, both a negative and solvent control are needed. However, when using a volatile solvent (*e.g.*, acetone), and the solvent is evaporated to completeness as recommended in paragraph (f)(2)(i) of the OCSPP 850.1000 guideline, use of only one control (solvent) may be allowed provided the testing laboratory demonstrate to the Agency that the solvent control is functionally equivalent to a negative control. Factors to be considered when demonstrating functional equivalency are:

(A) Concentration of solvent in the test sediment after evaporation (*e.g.*, analytically determined);

(B) Levels of the solvent that are known to affect organism health;

(C) The potential for impurities in the solvent and their potential impact on organism health and;

(D) Historical organism performance of solvent vs. negative controls.

(6) **Number of test organisms and replicates.** The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for the analysis of the data. See OCSPP 850.1000 for guidance. For tests designed to solely establish regression-based endpoints (LC_x, EC_x), at least three replicates per test concentration is recommended. For hypothesis or analysis of variance-based endpoints (NOEC), if a power analysis is not performed, at least eight replicates per test concentration should be used (see paragraph (j)(12) of this guideline). For pesticides, as the objective is to establish analysis of variance-based (NOEC) values, the minimum number of replicates per treatment is eight. For industrial chemicals, it is recommended that the Agency be consulted prior to test initiation to ascertain the appropriate study design to address the concerns for the test substance.

The recommended number of organisms in each replicate is 10.

Each test vessel should contain an equal amount of sediment and overlying water and an equal number of test organisms. In addition to the recommended minimum number of test vessels for conducting the effects analysis, separate replicate test vessels should be set up which can be destructively sampled for test substance concentrations during the test as described in paragraph (e)(9) of this guideline. Replicate test vessels should be physically separated, since the test vessel is the experimental unit.

(i) **Loading.** The number of test organisms placed in a test vessel should not be so great as to affect the results of the test. Experience has indicated that the use of 10 organisms in a test vessel containing 100 mL of sediment and 175 mL of overlying water which is renewed at least twice per day is acceptable. The loading should not cause the dissolved oxygen to fall below the recommended level of 2.5 mg/L or un-ionized ammonia to rise to levels that negatively impact organism health.

(ii) **Introduction of test organisms.** The test is initiated by adding the test organisms (either amphipods or midge larvae) to the test vessels which contain test sediment and overlying water. Test organisms should be introduced as soon as possible following their collection to minimize handling stress and exposure to temperature changes. Organisms are selected impartially and added singly or in small groups in a small volume of water, generally using a tube or dropper with a small suction bulb. The bore of the tube should be sized such that it is large

enough to transfer the organisms without damaging or excessively stressing the organisms. Amphipods or chironomids should be introduced directly into the overlying water either below the air-water interface or with a small amount of water pipetted directly into the exposure vessel just above the water surface. Care should be taken to observe the release of individual organism into the overlying water, insuring that organisms are not sticking to the transfer tube or becoming trapped in the surface tension; backlighting exposure beakers during transfer may aid these observations. When organisms are introduced below the air-water interface, separate transfer pipettes may be needed for different treatments to minimize potential cross contamination, not only of other exposure vessels but of the pool of organisms not yet assigned. Test vessels for treatment levels are randomly or indiscriminately located within the test area (see OCSPP 850.1000).

(7) **Facilities, apparatus and supplies.** Normal laboratory equipment should be used, especially the following:

(i) **Facilities.** Facilities for culturing or holding and acclimating and testing amphipods or midges that are well ventilated and free of fumes and disturbances which may affect the test organisms. Equipment for culturing and/or handling of food sources for amphipods or midges. Drying and ashing ovens, aluminum weighing pans, and an analytical balance capable of accurately weighing to 0.01 mg.

Reproduction/oviposit chambers with emergence traps for midges. Aspirator to collect and transfer midge adults from the reproduction/oviposit chambers.

(ii) **Environmental control equipment.** Mechanisms for controlling and maintaining the water temperature and lighting during the culturing, holding, acclimation, and test periods.

(iii) **Water quality testing instruments.** Equipment and instruments for determination of water quality and sediment quality characteristics (pH, hardness, temperature, *etc*.). For monitoring water and sediment quality, the use of instruments that do not require removal of water or sediment samples is recommended. Both dissolved oxygen and pH may be measured in overlying water using a probe. Probes should be thoroughly inspected between samples to make sure that organisms are not attached.

(iv) **Cleaning of test system.** Test vessels and overlying water delivery systems should be cleaned before each test. See OCSPP 850.1000 for further information. During the test, daily brushing of the outside of the screens on the test vessels is recommended.

(v) **Test containers and delivery system.** Construction materials and equipment that may contact the stock solution, sediment, or overlying water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Construction materials and equipment

that contact matrices containing test substances should be chosen to minimize sorption of test substances. Refer to OCSPP 850.1000 for additional information on appropriate construction materials. Test vessels, which should be constructed of chemically inert material, should be of a capacity to maintain the recommended loading level and environmental conditions. Test vessels consisting of 300 mL high-form lipless beakers (with notches and screens for overflow) have been used successfully. Test vessels should be loosely covered to reduce the loss of test solution or overlying water due to evaporation and to minimize entry of dust and other particles into the solutions. The system should contain appropriate test vessels in which to expose amphipods or midges to the test substance and an appropriate intermittent flow-through system. If renewal of the overlying water is to be automated, an appropriate delivery system is necessary. The overlying water delivery system should be monitored daily to assure proper operation.

(vi) **Overlying water.** Clean surface or ground water, or reconstituted water are acceptable as overlying water if it allows satisfactory survival and growth of the test organisms for the duration of the culturing and testing periods without showing signs of stress. Dechlorinated tap water is not recommended because some forms of chlorination are difficult to remove adequately. If dechlorinated tap water is used, recommended maximum chlorine levels as well as other ways to demonstrate suitability as a dilution water source are in OCSPP 850.1000. Reconstituted or natural water is preferred, although problems have been observed with the use of reconstituted water in long-term exposures with *H. azetca* (see paragraph (j)(12) of this guideline). However, several laboratories have successfully used the formulation from Borgmann (1996). Historically, a type of reconstituted water that has been used successfully in 10-day round-robin testing with *H. azteca* and *C. dilutus* is described in paragraph (j)(9) of this guideline. It is noted, however, that modifications to dilution water with respect to chloride and bromide concentrations have been recommended to improve performance for 10-d and chronic (life cycle) testing with *H. azteca*.

Dissolved oxygen in the dilution (overlying) water (prior to use in a test) should be between 90 and 100% saturation. If necessary, the overlying water can be aerated before it is introduced into the test vessels.

The pH should be between 6.0 and 8.5. For *C. dilutus* survival is best above pH 6.5, poor control survival in *C. dilutus* occurs at pH<6.5.

Where the source of overlying water is reconstituted water, the conductivity, hardness, and alkalinity should be measured in each batch.

Measurement of TOC or chemical oxygen demand (COD) in the overlying water source is recommended, but at a minimum TOC and COD should be measured periodically in the water source to document and characterize their magnitude and variability. For tests with cationic substances TOC or COD should be measured at the beginning of the test.

For *H. azteca*, the bromide concentration should be greater than or equal to 0.02 mg BR/L. NaBr can be added to reach this level. The chloride concentration should be greater than or equal to 15 mg Cl/L. NaCl can be added to reach this level.

(vii) **Sediment.** *H. azteca* can tolerate a wide range of substrates. Survival and growth in 10-d tests have not been shown to be negatively affected by either particle size (>90% silt and clay particles to 100% sand-sized particles) or organic matter. *C. dilutus* can tolerate a wide range of grain sizes and organic matter content. Growth may also be impacted by coarser sediment. More details on sediment conditions can be found in paragraphs (j)(1) and (j)(12) of this guideline. For pesticide testing, the use of formulated sediment as described in OCSPP 850.1000 (c)(4)(i) is preferred as long as the sediment supports the necessary survival and growth of the test organisms to meet study performance criteria. If natural sediment is used for testing of pesticides, it should be free of contaminants and organisms that may interfere with the test and should be typical of sediments naturally encountered by the test organism. For testing of industrial chemicals, the use of natural sediment or formulated sediment depends on the objective of the study; it is recommended that the submitter consult the Agency prior to study initiation to ascertain the appropriate sediment to use to address the concerns about the test substance. The characteristics of all sediment should be determined; specific characteristics are described in OCSPP 850.1000.

(8) **Environmental conditions.** Environmental parameters during the test should be maintained as specified below. The number and frequency of measurements recommended for documenting and confirming the magnitude and variability of water quality parameters (*e.g.*, temperature, dissolved oxygen, and pH) in the overlying water and sediment during the test are described in detail in OCSPP 850.1000.

If removing overlying water samples, they should be removed with a pipette from 1 to 2 cm above the sediment surface without disturbance. Caution is needed to avoid removing test organisms when sampling. Checking the pipette to make sure no organisms are removed during sampling of overlying water is recommended. Both dissolved oxygen and pH may be measured in overlying water using a probe.

To allow for measurements in sediment, these measurements may be made from the separate replicates resembling the biological replicates used to provide the required sample for chemical analysis.

(i) **Temperature.** The water temperature should be 23 °C. During a given test, the temperature should be constant within plus or minus (±) 1 °C. The mean and instantaneous temperatures should not vary from the desired temperature by more than 1 and 3 °C, respectively.

(ii) **pH**. The pH of the overlying water should be between 6.0 and 8.5 and vary less than 1 pH unit during the test within a test vessel and between test concentrations (including control(s)).

(iii) **Light.** A photoperiod should be selected from regimes of 12 hours light:12 hours dark to 16 hours light:8 hours dark. For any given test, the light regime should be constant. Light intensity should range from 540 to1080 lux (approximately 50-100 foot-candles (ft-c)). A 15- to 30-minute transition period between light and dark is suggested.

(iv) **Dissolved oxygen in overlying water.** Dissolved oxygen in the overlying water should be maintained above 2.5 mg/L. If dissolved oxygen falls below 2.5 mg/L in the overlying water in any one treatment, aeration is recommended and should be done in all replicates for the duration of the test. Aeration should be gentle enough that it does not disturb or resuspend sediment. If aeration is added, all test vessels should be checked daily for air flow to overlying water.

(v) **Ammonia and sediment Eh.** Ammonia concentrations in overlying water should be measured on day 0, and again on day 10. Ammonia should also be measured in the pore water and the beginning, mid-test, and end of the test. The sediment redox potential (Eh) should be measured at test initiation, mid-test (day 5), and at test termination.

(vi) **Flow in an intermittent flow-through system.**

(A) Water renewal systems should be designed such that volumes delivered to each exposure chamber do not differ by more than about 10%.

(B) Minimum number of test vessel volume replacements should be 2 per 24-hour period. If low dissolved oxygen requires action, the frequency/volume of overlying water addition can be increased from 2 up to a maximum of 4 volume additions/day.

(9) **Observations**

(i) **Measurement of test substance.** As described in OCSPP 850.1000, analytical confirmation of test substance concentrations should be performed on a subsample of each batch of spiked sediment for each test group to ensure that spiking is uniform. In addition, for pesticides with an aerobic soil or aerobic aquatic metabolism half-life of ≤10 days, analytical confirmation of test substance concentrations, and concentrations of major degradation products, if applicable, is performed in bulk sediment, interstitial water, and overlying water at a minimum at the beginning and end of the test in each test group. Analytical confirmation at the middle of the test (*i.e.*, day 5) is also recommended. For longer-lived pesticides and industrial chemicals, sampling at the beginning and end of the test in each test group may be appropriate. Sampling frequency should be adequate to characterize exposure concentrations in the bulk sediment and interstitial water throughout the duration of the test.

Measurements in interstitial water and overlying water should be of the dissolved or bioavailable form (see OCSPP 850.1000) whereas in bulk sediment the analytical measure is the total form. Separate test vessels should be set up at the

beginning of the test which can be destructively sampled during the test. The test vessels for test substance analytical measurements are set up and treated in the same way as those used for biological observations, including the presence of test organisms, except no biological observations are made. The analytical methods used to measure the amount of test substance in a sample should be validated before beginning the test, as described in OCSPP 850.1000.

The concentration of test substance in overlying water is measured by pipetting water samples from 1 to 2 cm above the sediment surface. Caution should be used to eliminate the presence of any surface debris, material from the sides of the vessel, or sediment in the overlying water sample. Measurement of test substance concentration in sediment can be taken by siphoning most of the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the bulk sediment for chemical analysis. The suggested method for isolation of interstitial water from a bulk sediment sample is by centrifugation without filtration. Centrifugation at about 10,000 g and 4 °C for 30 minutes is the recommended procedure to isolate interstitial water. In some cases pooling replicates may be necessary to analyze concentrations in the pore water. When pooling, the volume of interstitial water from each replicate should be equal.

(ii) **Overlying water and sediment appearance.** Observations are made daily on appearance of overlying water and test sediment. The appearance of surface slicks, precipitates, mold or fungus on sediment, or material adhering to the sides of the test vessels or in any part of the overlying water delivery or outflow system should be recorded.

(iii) **Measures of effect**

(A) **Monitoring of test organisms.** All test vessels should be checked daily. Any test organisms trapped in the air-water interface should be gently pushed back down using a glass rod or pipette. Test organisms should be observed for abnormal behavior, such as sediment avoidance. However, the test organisms are often not visible during the exposure. Any dead test organism observed on the surface of the sediment during the test should be counted, recorded, and removed.

(B) **Survival.** At test termination, all test organisms are collected, and the number of living, dead, and missing organisms is determined for each replicate in each test group. Amphipods are collected from the overlying water and the sediment surface of a test vessel by pipette prior to sieving the sediment for them. Surviving midges may be isolated from the sediment surface or from sieved material.

The basic method for obtaining amphipods and midges from test sediments is by sieving all contents of a test vessel through a U.S. Standard #40 sieve (425-µm opening) using a gentle stream of water. Material retained by the sieve is then rinsed into a sorting tray of clear or

translucent material, and surviving organisms are located and counted. Some sediments have large amounts of material too large to pass through a #40 sieve, resulting in a lot of debris in the sorting trays, which can hinder efforts to locate surviving organisms. In such cases, a procedure described by Kemble et al. (1994) may be effective. Rather than transfer the entire contents of the test vessel into the sieve, about half of the overlying water is decanted into the sieve, the remaining water is swirled vigorously enough to suspend the layer of sediment (roughly 1 cm), then the liquid portion is quickly dumped into the sieve, retaining a large portion of the sediment in the test vessel. The contents of the sieve are then washed, transferred to a sorting tray, and the surviving organisms isolated. Then the remaining sediment in the test vessel is sieved and examined for additional organisms in any remain. Initially sieving only a portion of the test vessel as described reduces the amount of debris in the sorting tray and makes it easier to locate the organisms; in most cases, most or all of the organisms are recovered in the initial effort.

Immobile organisms isolated from the overlying water, the sediment, or sieved material are considered dead. The amount of time taken to recover test organisms should be consistent for each replicate.

(C) **Growth**

For amphipods, growth may be measured either by weight or length. In this test design the primary determinate of growth is dry weight (measured to the nearest 0.01 mg). AFDW is an alternative measure of weight. Length to the nearest 0.1 mm is an optional measure of growth.

> *(1)* **Dry weight.** Dry weight is the primary determinate of growth in this test. To determine total dry weight for a replicate, all living test organisms from a given replicate are pooled together into a tared weigh pan and dried for at least 24 hours at about 60 ºC to a constant weight. The sample is brought to room temperature in a desiccator and weighed to the nearest 0.01 mg. It is important that the amphipods be free of all extraneous debris before being transferred to a weighing pan. One way to remove debris is to transfer amphipods from the sorting tray into a second sorting tray containing only clean water (generally the same as the overlying water used in the test). This transfer separates amphipods from the bulk of sediment debris (or sand) and usually makes it easier to see any debris still clinging to (or clung to by) the amphipods. Care should be taken to remove excess water from the weigh pan so that salts dissolved in the water do not overly influence the dry weight determination. As an example, 1 mL of the reconstituted water described by Borgmann (1996) would contain about 0.2 mg of dissolved ions, which could represent a meaningful proportion of the total dry

weight of amphipods being measured. If weight is to be measured following measurements of length in samples preserved in sugar formalin, care should be taken to thoroughly rinse the sugar formalin from the preserved *H. azteca* before weighing these organisms.

(*2)* **Ash free dry weight (AFDW).** Studies have indicated that the ash content of *H. azteca* is not greatly decreased by purging organisms in clean water before weighing, suggesting that sediment (*i.e.*, gut contents) does not comprise a large portion of the overall dry weight (T.D. Dawson, Badger Technical Services, Duluth, MN, unpublished data). Therefore, dry weight has remained the recommended endpoint for evaluating growth of *H. azteca*. However, the accuracy of dry weight for reflecting organism growth is dependent on avoiding any transfer of sediment particles to the weigh pan. Even a single grain of sand can be a significant influence on the dry weight measurement. To avoid inclusion of mineral sediment particles in weight determination, dried samples may be further subject to ashing at 500 to 550 C^{o} for 2 hours, with subsequent determination of ash free dry weight as the difference between dry weight and ash weight.

(*3*) **Length.** If both dry weight and body length measurements are to be determined, body length measurements on preserved samples need to be made before the drying procedure is initiated. Amphipods collected for length determinations should be preserved in an 8% sugar formalin solution or a similar preservative which will not result in deformation or significant distortion of the amphipod while in storage prior to length measurements. Preservation of amphipods in a 10% formalin solution for at least up to 4 weeks has been found not to significantly affect amphipod body length results. Body length is measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsum to the nearest 0.1 mm using an image analyzer.

For midges, ash-free dry weight (AFDW) is the measure of growth. Length measurements are an optional additional measurement of growth.

(*1*) **Ash-free dry weight.** AFDW is used rather than dry weight because the grain size of sediments has been found to influence the amount of sediment that the larvae ingest and retain in their gut, and thus, in fine-grained sediments, a substantial portion of the measured dry weight may be comprised of sediment rather than tissue. This may not represent a strong bias in tests with identical grain size distributions in all treatments, as would be the

case in the use of formulated sediment. Nonetheless, the AFDW procedure is recommended. All surviving larvae in a replicate should be pooled and placed into a weigh boat and dried at about 60-90 °C to a constant weight. (Weigh boats should be ashed and weighed prior to use.) The sample is brought to room temperature in a dessicator and weighed to the nearest 0.01 mg ($Dry\ Weight_{(pan\ +\ larvae)}$). The dried larvae in the weigh pan are then ashed at 550 °C for 2 hours. The pan with the ashed larvae is removed from the oven and cooled to room temperature and then re-weighed ($Ash\ Weight_{(pan+ash)}$). The AFDW total tissue mass of the larvae is determined as the difference in weight between the dry weight of the larvae plus pan and the ashed weight of the larvae plus pan (Equation 1).

$$Total\ AFDW = Dry\ Weight_{(pan+larvae)} - Ash\ Weight_{(pan+ash)} \quad \textbf{Equation 1}$$

(*2*) **Length.** Measurements should be from the anterior of the labrum to the posterior of the last abdominal segment. Length may be determined from midges kept in separate replicate beakers that were prepared at the beginning of the test. An 8% sugar formalin solution can be used to preserve samples for length measurements.

(*3*) **Head capsule width.** If head capsule width is to be measured for midges, it should be measured on surviving midges at the end of the test before AFDW is determined.

Biomass. Biomass is calculated as the total mass (dry weight or AFDW as applicable) of all organisms recovered from a test vessel. Biomass is an optional endpoint that may be reported for amphipods or midges in addition to dry weight and/or length.

(f) **Treatment of results.**

(1) **Response variable calculation.** For measures of survival, surviving organisms are counted at test termination. For measures of growth, the response variables used in the analyses are the average weight, length, and/or head capsule width (for the midge only) of surviving test organisms.

Average test organism weight. The response measure for test organism weight for a test vessel is the average test organism weight ($\overline{W}_j$) which is calculated using Equation 2.

$$\overline{W}_j = {TW_j}/{NS_j} \quad \textbf{Equation 2}$$

where:

j = index number of the replicates in a test group from 1 to the total number of test vessels in a test group;

TW_j = total weight of surviving test organisms (dry weight for amphipods measured as described in paragraph (e)(9)(v)(C) and AFDW for midges measured as described in paragraph (e)(9)(v)(C)) in replicate j; and

NS_j = number of surviving test organisms in replicate j (that were weighed).

Average test organism length. The response measure for test organism length is the average test organism length for a test vessel ($\overline{L}_j$) which is calculated using Equation 3.

$$\overline{L}_j = \sum_{k=1}^{NS_j} L_{kj} \Big/ NS_j \qquad \textbf{Equation 3}$$

where:

j = index number of the replicates in a test group from 1 to the total number of test vessels in a test group;

k = index number of length measurement in test vessel j from 1 to NS_j; and

NS_j = number of surviving test organisms in replicate j that were measured.

Average head capsule width. If head capsule width was measured for midges, calculate the average head width for a test vessel ($\overline{C}_j$) which is calculated using Equation 3.

$$\overline{C}_j = \sum_{k=1}^{NS_j} C_{kj} \Big/ NS_j \qquad \textbf{Equation 3}$$

where:

j = index number of the replicates in a test group from 1 to the total number of test vessels in a test group;

k = index number of head capsule measurements in test vessel j from 1 to NS_j; and

NS_j = number of surviving test organisms in replicate j that were measured.

(2) **Summary statistics**

(i) **Environmental conditions.** For overlying water, interstitial (pore) water, and sediment homogeneity:

(A) **Overlying water.** Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) of environmental conditions (temperature, dissolved oxygen, pH, and un-ionized ammonia) monitored during the test.

(B) **Interstitial (pore) water.** Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) of interstitial (pore) water environmental conditions (Eh, pH and un-ionized ammonia) monitored during the test.

(C) **Sediment homogeneity.** Calculate descriptive statistics (mean standard deviation, coefficient of variation, minimum, maximum) of bulk sediment characteristics, such as TOC, particle size distribution (percent of sand, silt, clay), percent water content, and acid volatile solids, if applicable.

(ii) **Test substance concentration**

(A) **Overlying and Interstitial water.** Calculate descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation) by test vessel and treatment level of the test substance soluble concentration in overlying water and interstitial water.

(B) **Bulk sediment.** Calculate descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation) by test vessel and treatment level of the test substance concentration in bulk sediment. Where applicable calculate descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation) by test vessel and treatment level of the major toxic degradate(s) concentration in bulk sediment. Test concentrations are typically expressed as mg/kg dry weight for sediment. In some cases, it is also desirable to normalize sediment concentrations of test substance to factors other than sediment dry weight, such as organic carbon content for nonionic organic compounds or acid volatile sulfides for certain metals.

(iii) **Mortality.** Number of organisms exposed at test initiation in each treatment and replicate and the cumulative number of dead test organisms should be summarized in tabular form by time of observation, treatment, and replicate.

(iv) **Growth**

(A) **Weight and length.** Average weight and/or length of surviving test organisms at test termination by treatment and replicate. The average dry weight of surviving test organisms is used for amphipods and the average

AFDW of surviving test organism is used for midges. Length is used as an additional optional measure of growth for amphipods and midges.

(B) **Head capsule width.** If head capsule measurements were made on midges at test termination, average head capsule width of surviving test organisms by treatment and replicate.

(v) **Sediment Avoidance.** Number of organisms appearing to avoid the sediment (*e.g.*, observed in overlying water or on top of the sediment when should be under cover) should be summarized in tabular form by time of observation, treatment, and replicate.

(vi) **Appearance and behavior.** Number of organisms exhibiting abnormal appearance or behavioral symptoms should be summarized in tabular form time of observation, treatment, and replicate.

(3) **Percent mortality.** Calculate the percent mortality at each treatment level and in the control(s) at test termination.

(4) **Percent inhibition.** For survival and growth (weight and/or length) calculate and plot the mean percent inhibition for survival and growth at test termination in each treatment level as compared to the negative sediment control.

(5) **Evaluation of limit test results.** To ascertain that there is no observable effect at the limit concentration (*i.e.*, NOEC>limit concentration) for a given response measure (survival or growth as measured by weight and/or length), the limit treatment response is compared to the control treatment response. For pesticides, if a significant effect is detected in survival or growth at the limit concentration, it is preferred that a multiple-concentration sub-chronic 10-d test be conducted. For industrial chemicals, if the effect level for mortality or inhibition of growth at the limit concentration compared to the control(s) is 50% or greater, then a definitive test should be conducted.

(6) **Evaluation of multiple-concentration definitive test**

(i) **Concentration-response curve, slope, and LC_{50} and EC_{50}.** Statistical procedures are employed to calculate the 10-d LC_{50} (standard error and 95% confidence interval) based upon mortality and 10-d EC_{50} (standard error and 95% confidence intervals) for growth (weight and/or length) when appropriate to the test design. The slope of the concentration-response curve, its standard error, and 95% confidence interval should also be reported. If a concentration-response curve model was fit to the data to determine the LC_{50} or EC_{50}, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded.

(ii) **NOEC.** Calculate the NOEC and LOEC values for survival and growth (weight, length) when appropriate to the test design. Hypothesis testing procedures described in OCSPP 850.1000 can be used to determine NOEC and LOEC values.

(iii) **Statistical methods.** Statistical procedures for modeling quantal data should be used for calculating the LC_{50}. Statistical procedures for modeling continuous toxicity data should be used for calculating the EC_{50} (see references in paragraphs (j)(3) and (j)(6) of this guideline). Analysis-of-variance testing procedures are used to determine NOEC and LOEC values. Additional discussion about endpoints and statistical procedures is found in OCSPP 850.1000 and in paragraphs (j)(1) and (j)(12).

(g) **Tabular summary of test conditions.** Table 1 and Table 2 list the important conditions that should prevail during this test for *H. azteca* and *C. dilutus*, respectively.

Table 1.—Summary of Test Conditions for the 10-Day Whole Sediment Toxicity Test with *H. azteca*

Test type	Spiked-sediment toxicity test
Test duration	10 days
Test matrix	Formulated or natural clean sediment with overlying water
Overlying water	Surface water, well water, groundwater, or reconstituted water
Temperature	23ºC (constant within ±1 ºC during test)
Light quality	Ambient laboratory illumination
Light intensity	100-1080 lux (10-100 foot-candles)
Photoperiod	Selected from among 12 hours light:12 hours dark to 16 hours light:8 hours dark schemes
Overlying water pH	Between 6.0 and 8.5 (constant within ±1 pH unit within a test vessel and between test concentrations (including control(s)) during test
Overlying water hardness (as $CaCO_3$)	<250 mg/L (preferably <180 mg/L)
Overlying water TOC	≤2 mg/L
Sediment/overlying water volume	100 mL/175 mL in 300-mL high form beaker has been used successfully
Renewal of overlying water	2 volume replacements/day (intermittent flow-through or static renewal)
Age of test organisms	7- to 10-days old at test initiation (within 24 hour range in age for a given test)
Number of test organisms per test vessel	10
Number of replicate test vessels per concentration	For pesticides, minimum of 8 for NOEC/LOEC determination For industrial chemicals, minimum of 3 for LC_{50} and EC_{50} determinations Additional replicates may be needed for use in analytical determinations of test substance concentrations in bulk sediment, pore water, and overlying water and sediment chemistry during the test

Feeding regime	**Option 1:** Diatom+flake fish food Diatom suspension: 1. Day 0 to 6: 0.5 mg/beaker-day 2. Day 7 to 9: 0.75 mg/beaker-day Flake fish food suspension: 1. Day 0 to 6: 0.25 mg/beaker-day 2. Day 7 to 9: 0.5 mg/beaker-day **Option 2:** YCT+flake fish food YCT: 1.0 mL/beaker-day Flake fish food suspension: 1. Day 0 to 6: 0.25 mg/beaker-day 2. Day 7 to 9: 0.5 mg/beaker-day
Test vessel aeration	Not recommended unless dissolved oxygen drops below 2.5 mg/L
Test vessel cleaning	Gently brush outside of screen when clogged
Test concentrations	Unless performing a limit test, minimum of 5 test concentrations chosen in a geometric series plus appropriate control(s)
Test concentration preparation	Addition of the test substance directly to the sediment as an aqueous solution for a soluble test substance, directly to the sediment in a dry form for a water insoluble solid, or by sorbing the test substance to sand and then mixing the treated sand into the sediment for a water insoluble test substance
Measures of Effect or Measurement Endpoints	For pesticides, 10-d NOEC and LOEC for survival and growth; if desired, 10-d LC_{50} (mortality), 10-d EC_{50} (growth based on weight or length) For industrial chemicals, 10-d LC_{50} (mortality), 10-d EC_{50} (growth based on weight or length)

Table 2.—Summary of Test Conditions for the 10-Day Whole Sediment Toxicity Test with *C. dilutus*

Test type	Spiked-sediment toxicity test
Test duration	10 days
Test matrix	Formulated or natural clean sediment with overlying water
Overlying water	Surface water, well water, groundwater, or reconstituted water
Temperature	23ºC (constant within $\pm$1 ºC during test)
Light quality	Ambient laboratory illumination
Light intensity	100-1080 lux (10-100 foot-candles)
Photoperiod	Selected from among 12 hours light:12 hours dark to 16 hours light:8 hours dark schemes
Overlying water pH	Between 6.0 and 8.5 (constant within ±1 pH unit within a test vessel and between test concentrations (including control(s)) during test)
Overlying water hardness (as $CaCO_3$)	<250 mg/L (preferably <180 mg/L)
Overlying water TOC	$\leq$2 mg/L
Sediment/overlying water volume	100 mL/175 mL in 300-mL high form beaker has been used successfully

Renewal of overlying water	2 volume replacements/day (intermittent flow-through or static renewal)
Age of test organisms	Second- to third-instar larvae (about 10-d old larvae; with at least 50% of the organisms at or below the third instar)
Length of test organisms at test initiation	Average length of 4-6 mm
Number of test organisms per test vessel	10
Number of replicate vessels per concentration	For pesticides, minimum of 8 for NOEC/LOEC determination For industrial chemicals, minimum of 3 for LC_{50} and EC_{50} determinations Additional replicates may be needed for use in analytical determinations of test substance concentrations in bulk sediment, pore water, and overlying water and sediment chemistry during the test)
Feeding regime	Daily, 1.5 mL of fish flake food suspension (~4.0 g/L stock) to each test vessel
Test vessel aeration	Not recommended unless dissolved oxygen drops below 2.5 mg/L
Test vessel cleaning	Gently brush outside of screen when clogged
Test concentrations	Unless performing a limit test, minimum of 5 test concentrations chosen in a geometric series plus appropriate control(s)
Test concentration preparation	Addition of the test substance directly to the sediment as an aqueous solution for soluble test substances, directly to the sediment in a dry form for a water insoluble solid, or by sorbing the test substance to sand and then mixing the treated sand into the sediment for water insoluble test substances
Measures of Effect or Measurement Endpoints	For pesticides, 10-d NOEC and LOEC for survival and growth; if desired, 10-d LC_{50} (mortality), 10-d EC_{50} (growth based on weight, length, or head capsule width) For industrial chemicals, 10-d LC_{50} (mortality), 10-d EC_{50} (growth based on weight, length, or head capsule width)

(h) Test validity elements. This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 3 and Table 4 occurred for *H. azteca* and *C. dilutus*, respectively. These parameters are not the only elements considered when evaluating the acceptability of a test, and it is possible that a test could be found unacceptable or invalid based on other considerations. However, except for the conditions listed in Table 3 and Table 4 and in OCSPP 850.1000, it is unlikely that a study will be rejected when there are only slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline (such as deviating from the organism age), the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 3.—Test Validity Elements for the 10-Day Whole Sediment Toxicity Test with *H. azteca*

1. All test vessels were not identical or did not contain the same amount of sediment and overlying water.

2. Test organisms were not randomly or impartially assigned to test vessels.

3. A negative sediment control and a solvent sediment control, if applicable, were not included in a test.

4. Average survival of *H. azteca* in the either the negative sediment control or solvent sediment control was not greater than or equal to 80% at test termination.

5. Average dry weight of test organisms in either the negative sediment control or the solvent sediment control, if applicable, did not exceed 2.5x the initial dry weight measured at the start of the test.

Table 4.—Test Validity Elements for the 10-Day Whole Sediment Toxicity Test with *C. dilutus*

1. All test vessels were not identical or did not contain the same amount of sediment and overlying water.

2. Test organisms were not randomly or impartially assigned to test vessels.

3. A negative sediment control and a solvent sediment control, if applicable, were not included in a test.

4. Average survival of *C. dilutus* in the either the negative sediment control or solvent sediment control, if applicable, was not greater than or equal to 70% at test termination.

5. Average mass of *C. dilutus* in the negative sediment control or solvent sediment control, if applicable, was not at least 0.48 mg AFDW at the end of the test.

(i) **Reporting**

(1) **Background information.** Paragraph (k)(1) of OCSPP 850.1000 describes the minimum background information to be supplied in the report.

(2) **Guideline deviations.** Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test, the reasons for these changes, and any resulting effects on test endpoints noted and discussed.

(3) **Test substance**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and

concentration of active ingredient(s)), radiolabeling if any, location of label(s), and radiopurity.

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods of preparation of the test substance and the treatment concentrations used in the range-finding and definitive test, or limit test. Identify whether the nominal concentrations are corrected or uncorrected for purity of the test substance.

(iv) Physicochemical properties of the test substance such as: water solubility, vapor pressure, UV absorption, pKa, K_{ow}.

(v) If a vehicle (solvent) is used to prepare stock or test substance, provide: the name and source of the solvent, the nominal concentration(s) of the test substance in the solvent in stock solutions or mixtures, and the solvent concentration(s) used in the treatments and solvent sediment control. If different solvent concentrations are used at different treatment levels, the report should, at a minimum, identify the maximum solvent concentration used. It is helpful to support the solvent choice by including a description of any measures that were taken to identify an appropriate solvent for use in the study, such as the types and concentrations of solvents used and their corresponding effects on solubility during any preliminary work.

(vi) If a positive control is used, provide: the name and source of positive control and the nominal concentration(s) of the positive control material in stock solutions or mixtures.

(4) **Test organism**

(i) Scientific name and common name.

(ii) Method for verifying the species.

(iii) Information about the organisms used in the test: source, date of collection, duration of quarantine for organisms collected from a natural population, culture practices, and holding and acclimation procedures and conditions, including environmental conditions, acclimation period, water used, substrate, feeding history, and health status of culture used to obtain larvae or instars (mortality of stock before test initiation and any preventative or disease treatments).

(iv) Age (days, developmental stage) and size (weight, length, head capsule width (for midges)) of test organisms in the test population at test initiation.

(5) **Test system and conditions.** Provide a description of the test system and conditions used in the definitive or limit test, and any preliminary range-finding tests.

(i) Description of the test container used: size, type, material, fill volume.

(ii) For naturally derived sediment, description of the sediment source and procedures for collection and handling: location, time, core depth, water depth, collection equipment, shipping method, pooling, pretreatment (sieving, UV treatment), and storage methods and duration.

(iii) For an artificial sediment, description of the source, type and amounts of ingredients used to prepare formulated sediment, preparation date, mixing and homogenization procedures, and storage methods and duration.

(iv) Type and magnitude of biota present in naturally-derived sediment at test initiation.

(v) Physical and chemical characteristics of the bulk sediment: color, pH, Eh, particle size distribution (percent sand, silt, clay), TOC, cation exchange capacity, moisture content (percent water content of the sediment), biological oxygen demand (BOD), and COD of the sediment. If from a naturally-derived source, the background concentration of metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and ammonia. If metals are tested, the concentration of acid volatile sulfides should also be reported. Describe the frequency and sampling methods and sampling date(s) for documenting physical and chemical characteristics and the homogeneity of the sediment physical and chemical characteristics.

(vi) Description of the overlying water renewal technique used: static renewal or intermittent flow-through and the rate of renewal. If static renewal, the frequency of test solution renewal, and if intermittent flow-through, a description of the flow-through system, including flow rates and test vessel turnover rate. For closed systems, a description of the closed system design. For all systems, a description of the calibration and validation methods used.

(vii) Description of the source of clean water used for overlying water and any water pretreatment: source/type; temperature; pH; hardness and alkalinity; total organic carbon or chemical oxygen demand; particulate matter; conductivity; metals, pesticides, and residual chlorine concentrations (mean, standard deviation, range). Describe the frequency and sample date(s) for documenting source water quality and consistency.

(viii) Methods of adding the test substance to sediment and concentrations used in definitive or limit testing.

(ix) Equilibration phase of the spiked sediment-water system: duration and conditions.

(x) Depth and volume of sediment and overlying water in test vessels.

(xi) Use of aeration, if any, and method.

(xii) Number of test organisms added to each test vessel at test initiation and the method of introduction.

(xiii) Number of test vessels (replicates) per treatment level and control(s).

(xiv) Methods used for treatment randomization and assignment of test organisms to test vessels.

(xv) Date of introduction of test organisms to test vessels and test duration.

(xvi) The photoperiod and light source and quality.

(xvii) Description of feeding protocols: source and composition of food; preparation of food; frequency of feeding and food ration; frequency and sample date(s) for documenting the contaminant status (heavy metals, persistent or chlorinated pesticides) of the feed and tabulation of the results of the analysis.

(xviii) Methods and frequency of environmental monitoring performed during the definitive or limit study for overlying water temperature, dissolved oxygen, pH, ammonia, conductivity, and alkalinity.

(xix) Methods and frequency of monitoring performed during the definitive or limit study for pore water pH and ammonia.

(xx) Methods and frequency of monitoring performed during the definitive or limit study for sediment Eh.

(xxi) Methods and frequency of measuring test substance (and major degradates where appropriate) in bulk sediment, pore water, and overlying water to verify exposure concentrations.

(xxii) Methods and frequency of counting number of surviving and dead test organisms and measuring dry weight or AFDW, length, head capsule width, and sediment avoidance and any other toxic symptoms.

(xxiii) For definitive and limit tests, a description of all analytical procedures used, accuracy of the method, method detection limit, and limit of quantification.

(6) **Results**

(i) Nominal sediment exposure concentrations and a tabulation of test substance analytical results for bulk sediment, pore water, and overlying water by treatment group and test vessel (provide raw data) and descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation). For testing with organic compounds, also provide a tabulation of test substance sediment analytical results normalized to organic carbon content of sediment. For testing with certain types of metals, also provide a tabulation of sediment analytical results normalized to acid volatile sulfide content of sediment.

(ii) Environmental monitoring data results in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

(iii) For preliminary range-finding tests, if conducted, a tabulation of the number and percentage of organisms that died in each test vessel, including all treatment levels and control(s), at each observation period. A description and count of any test organisms exhibiting avoidance or other appearance or behavioral effects at each treatment level and in the control(s).

(iv) For limit and definitive tests, if conducted, a tabulation of:

(A) The number of dead test organisms and the percent mortality in each test vessel, including all treatment levels and control(s), at test termination (provide the raw data) and descriptive statistics (mean, standard deviation, minimum, maximum).

(B) The number of organisms exhibiting avoidance behavior or other abnormal behavior or appearance by test vessel, treatment level, and observation period (provide the raw data).

(C) The dry weight or length for *H. azteca* and the AFDW and length (optional) for *C. dilutus* in each vessel, including all treatment levels and control(s), at test termination (provide the raw data) and descriptive statistics (mean, standard deviation, minimum, maximum).

(D) The percent reduction in survival and percent inhibition in growth (weight, length) for the limit concentration as compared to the control(s).

(v) Graphs of the concentration-response data for percent mortality, percent inhibition of in survival, and percent inhibition in growth (length, weight).

(vi) For limit tests, conclusion about the 10-d NOEC for survival and growth (weight, length).

(vii) For definitive tests with industrial chemicals, the 10-d LC_{50} and EC_{50} value, its standard error and 95% confidence interval, and where sufficient data exist to fit a regression model (*e.g.* probit), the slope of the concentration-response curve, its standard error and 95% confidence intervals, and any goodness of fit results for mortality.

(viii) For definitive tests with pesticides, the 10-d NOEC and LOEC for survival and growth (weight, length).

(ix) Description of statistical method(s) used for point estimates, including software package, for determining the LC_{50} and EC_{50} values, fitting the concentration-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(x) Description of statistical method(s) used for NOEC and LOEC determination, including software package, and the basis for the choice of the method.

(j) **References**. The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials. ASTM E-1706-05, Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates, American Society for Testing and Materials, West Conshohocken, PA. Current edition approved 2010. http://www.astm.org/Standards/E1706.htm.

(2) American Society for Testing and Materials. ASTM E-1391-03, Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates, American Society for Testing and Materials, West Conshohocken, PA. Current edition approved 2008. http://www.astm.org/Standards/E1391.htm.

(3) Bruce, R.D. and D.J. Versteeg, 1992. A statistical procedure for modeling continuous toxicity data. Environmental Toxicology and Chemistry 11:1485-1494.

(4) Environment Canada, 1997. Biological test method: Test for survival and growth in sediment using the freshwater amphipod *Hyalella azteca*, Environment Canada, Ottawa, Ontario, Report EPS 1/RM/33, December 1997.

(5) Environment Canada, 1997. Biological test method: Test for survival and growth in sediment using the larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius*), Environment Canada, Ottawa, Ontario, Report EPS 1/RM/32, December 1997.

(6) European Commission, 1994. Sediment toxicity tests for poorly water-soluble substances, final report to the European Commission, Report No. EC 3738, programme co-ordinator R. Fleming.

(7) Nyholm, N., P.S. Sorenson, K.O. Kusk, and E.R. Christensen, 1992. Statistical treatment of data from microbial toxicity tests. Environmental Toxicology and Chemistry 11:157-167.

(8) Shobanov, N.A., Kiknadze, I.I., and Butler, M.G., 1999. Palearctic and Nearctic *Chironomus* (*Camptochironomus*) *tentans* (Fabricius) are different species (Diptera: Chironomidae). Ent. Scand. 30:311-322 (Copenhagen, Denmark).

(9) Smith, M.E., J.M. Lazorchak, L.E. Herrin, S. Brewer-Swartz, and W.T. Thoney, 1997. A reformulated, reconstituted water for testing the freshwater amphipod, *Hyalella azteca,* Environ. Toxicol. Chem. 16:1229-1233.

(10) Society of Environmental Toxicology and Chemistry - Europe, 1994. Guidance document on sediment toxicity tests and bioassays for freshwater and marine

environments, from the Workshop on sediment toxicity assessment, held 8-10 November, 1993, Hill, I.R., Matthiessen,

(11) P. and Heimbach, F., eds. Streloke, M. and H. Köpp, eds., 1995. Long-term toxicity test with *Chironomus riparius*: development and validation of a new system, Biologische Bundesanstalt für Land- und Forstwirtschaft, Abteilung für Pflanzenschutzmittel und Anwendungstechnik, Braunschweig.

(12) U.S. Environmental Protection Agency, 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates, Second Edition, EPA 600/R-99/064, March 2000. http://www.epa.gov/waterscience/cs/freshfact.html.

(13) U.S. Environmental Protection Agency, 2014. Proposed Revisions to U.S. EPA (2000) Guidance for Sediment Toxicity and Bioaccumulation Test Methods for Freshwater Sediments. Prepared by U.S. EPA Office of Research and Development, National Health and Ecological Effects Laboratory, Mid-Continent Ecology Division, Duluth, MN, September 22, 2014. *In press*.