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Standard Guide for Conducting Whole Sediment Toxicity Tests with Amphibians[1]

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1. Scope

1.1 This standard covers procedures for obtaining laboratory data concerning the toxicity of test material (for example, sediment or hydric soil (that is, a soil that is saturated, flooded, or ponded long enough during the growing season to develop anaerobic (oxygen-lacking) conditions that favor the growth and regeneration of hydrophytic vegetation)) to amphibians. This test procedure uses larvae of the northern leopard frog (*Rana pipiens*). Other anuran species (for example, the green frog (*Rana clamitans*), the wood frog (*Rana sylvatica*), the American toad (*Bufo americanus*)) may be used if sufficient data on handling, feeding, and sensitivity are available. Test material may be sediments or hydric soil collected from the field or spiked with compounds in the laboratory.

1.2 The test procedure describes a 10-d whole sediment toxicity test with an assessment of mortality and selected sublethal endpoints (that is, body width, body length). The toxicity tests are conducted in 300 to 500-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and larval amphibians are fed during the toxicity test once they reach Gosner stage 25 (operculum closure over gills). The test procedure is designed to assess freshwater sediments, however, *R. pipiens* can tolerate mildly saline water (not exceeding about 2500 mg Cl^-/L, equivalent to a salinity of about 4.1 when Na^+ is the cation) in 10-d tests, although such tests should always include a concurrent freshwater control. Alternative test durations and sublethal endpoints may be considered based on site-specific needs. Statistical evaluations are conducted to determine whether test materials are significantly more toxic than the laboratory control sediment or a field-collected reference sample(s).

1.3 Where appropriate, this standard has been designed to be consistent with previously developed methods for assessing sediment toxicity to invertebrates (for example, *Hyalella azteca* and *Chironomus dilutus* toxicity tests) described in the United States Environmental Protection Agency (USEPA, **(1)**)[2] freshwater sediment testing guidance, Test Methods E1367 and E1706, and Guides E1391, E1525, E1611, and E1688. Tests extending to 10 d or beyond, and including sublethal measurements such as growth, are considered more effective in identifying chronic toxicity and thus delineating areas of moderate contamination **(1-3)**.

1.4 Many historical amphibian studies, both water and sediment exposure, have used tests of shorter duration (5 days or less) (for example, **4-7**) and, although both survival and sublethal endpoints were often assessed, there is substantive evidence that tests of longer duration are likely to be more sensitive to some contaminants **(8, 9)**. Research performed to develop and validate this test protocol included long-term (through metamorphosis) investigations and other researchers have also conducted long-duration tests with anurans **(7-11)**. In the development of these procedures, an attempt was made to balance the needs of a practical assessment with the importance of assessing longer-term effects so that the results will demonstrate the needed accuracy and precision. The most recent sediment toxicity testing protocols for invertebrates have encompassed longer duration studies which allow the measurement of reproductive endpoints **(1, 12)**. Such tests, because of increased sensitivity of the sublethal endpoints, may also be helpful in evaluating toxicity. Full life-cycle studies with anurans (including reproduction) are usually not feasible from either a technical or monetary standpoint. However, if site-specific information indicates that the contaminants present are likely to affect other endpoints (including teratogenicity), then the duration of the toxicity test may be increased through metamorphosis or additional sublethal endpoints may be measured (for example, impaired behavior, deformities, time-to-metamorphosis). The possible inclusion of these endpoints and extension of test length should be considered during development of the project or study plan (see 8.1.1).

1.5 The methodology presented in this standard was developed under a Department of Defense (DoD) research program and presented in a guidance manual for risk assessment staff

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

and state/federal regulators involved in the review and approval of risk assessment work plans and reports (**13**). To develop this method, a number of tests with spiked sediment tests were conducted (**13, 14**). Since development of the methodology it has been used operationally to evaluate field-collected sediments from several state and federal environmental sites (**15, 16**). For most of these studies the preferred test organisms, *Rana pipiens*, was used. At a lead-contaminated state-led site, operated by the Massachusetts Highway Department, *Xenopus laevis*(African clawed frog) was used in the sediment test system because of availability problems with *Rana pipiens* (**17**), The test method was also used to evaluate sediment toxicity at a cadmium-contaminated USEPA Region 4-led site in Tennessee (**18**). The methodology was used to help characterize potential effects of contaminants on amphibians and to help develop preliminary remedial goals, if warranted. All tests evaluated survival and growth effects after 10 d of exposure in accordance with the methods presented in this standard.

1.6 The use of larval amphibians to assess environmental toxicity is not novel. Researchers have used tadpoles to examine toxicity of metals and organic compounds. Most of these studies have been through water exposure, usually in a manner similar to fish or invertebrate exposure as described in Guide E729 (**19-29**). Fewer studies have focused on exposure of anuran larvae to sediments, and the methods employed vary widely, from *in situ* enclosures (**30**) to laboratory tests using variable exposure conditions and organism ages (**4, 8, 31-33**). No studies were identified that used the same test conditions as described in this standard. However, several laboratory-based evaluations of sediment effects on amphibians are described in the following subsections.

1.6.1 Sediment toxicity tests conducted in the laboratory with amphibians were performed over a range of test durations from 4 d (**4**, **31**, Guide E1439-98 Appendix X2) to 12 d (**33**) and through metamorphosis (**8, 32**). Sediment toxicity tests with anurans native to North America were started with larval tadpoles between Gosner stages 23 and 25 (**8, 32, 33**). Test temperatures were between 21 and 23°C and feeding began after tadpoles reached Gosner stage 25. Food sources were Tetramin™ (**8**), boiled romaine lettuce (**32**), or boiled romaine lettuce and dissipated rabbit food pellets (**33**). Tests were conducted in static renewal mode with water replacements conducted at varying rates (daily (**31, 33**), weekly (**8**), every 3 to 5 d (**32**)). Test design (number of replicates, test vessel size, number of organisms per replicate) varied depending on the objective of the study with several tests conducted in aquaria (**32**), large bins (**8**), or swimming pools (**33**). Endpoints evaluated at test termination included survival (**4, 8, 31-33**), growth (**8, 31-33**), bioaccumulation of metals (**8**), developmental rates (**8, 32**), deformities (**31, 32**), swimming speed (**33**) and foraging activity levels (**32**).

1.6.2 To assess the effect of direct contact with the sediments containing PCBs, Savage et al. (**32**) exposed larval tadpoles (Gosner stage 23 to 25; wood frogs (*R. sylvatica*)) to field-collected sediments under conditions that allowed both direct contact with the sediment and separation from the sediment with a 500 µm mesh barrier. The study found that lethal and sublethal effects on tadpoles observed through metamorphosis were more pronounced when direct contact with the sediment was allowed. The test conditions described in this standard allow tadpoles to maintain direct contact with the sediment.

1.6.3 Sediment toxicity testing with *Xenopus laevis* has focused on evaluating the developmental effects of sediment extracts, as opposed to whole sediments, on frog embryos. Methods have been developed which expose blastula stage embryos to sediment by enclosing the embryos in a Teflon mesh insert that rests over the top of the sediment in the sediment–water interface region (**31**, Guide E1439-98 Appendix X2). These studies are conducted evaluate survival, growth, and physical malformations of the embryos after a 4-d exposure period. The test conditions described in this standard allow more direct contact with the sediment, using older test organisms, and a longer exposure duration.

1.7 Sediment toxicity tests are an effective means for evaluating the impact of sediment contamination on amphibians in a multiple lines of evidence paradigm. The evaluation is most powerful when toxicity testing sampling stations are co-located with sediment analytical chemistry samples and ecological surveys, allowing for a detailed evaluation of the co-occurring data in the ecological risk assessment. The spatial and temporal co-location of toxicity testing and analytical samples is particularly important for establishing contaminant-specific effects and assessing contaminant bioavailability.

1.8 In order for a sediment toxicity test to be sensitive it must be of sufficient duration to measure potential toxicity and it must be conducted during the appropriate developmental stage of the test organism's life cycle. Using recently hatched tadpoles and conducting the sediment exposure test for 10 d to allow the evaluation of growth endpoints meets both of these sensitivity requirements.

1.9 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.10 *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 limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E729 Guide for Conducting Acute Toxicity Tests on Test

[3] 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.

Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates
E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates
E1439 Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)
E1525 Guide for Designing Biological Tests with Sediments
E1611 Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids
E1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates
E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

3. Terminology

3.1 The words "must", "should", "may", "can" and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the design of a test ought to be in a manner that satisfies the specified conditions, unless project goals dictate needed alterations in order to address the study hypotheses. "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although the violation of one "should" is rarely a serious matter, violation of several could render the results questionable. Terms such as "is desirable", "is often desirable" and "might be desirable" are used in association with less important factors, the alteration of which will probably not have substantive effects on test outcome. "May" means "is (are) allowed to," "can" means "is (are) able to" and "might" means "could possibly." In this manner, the classic distinction between "may" and "can" is preserved and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions*—For definitions of general terms related to toxicity testing and used in this guide, refer to Guide E943.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *IC25 (25 % inhibition concentration), n*—concentration at which there is a 25 % reduction in organism performance, relative to the control. Performance may be survival or a sublethal measurement such as growth.

3.3.2 *overlying water, n*—water that is placed over the sediment for the duration of the study. Overlying water may be surface water collected from the project site or from a clean lake or reservoir, or may be reconstituted water prepared in the laboratory (for example, moderately hard water; (**34**)).

3.3.3 *reference-toxicant test, n*—a test conducted with a reagent-grade reference chemical to assess the sensitivity of the test organisms. Deviations outside an established normal range may indicate a change in the sensitivity of the test organism population. Reference-toxicity tests are most often performed in the absence of sediment.

3.3.4 *test sediment or test material, n*—sediment that may contain contaminants, which is being evaluated using this test procedure.

4. Summary of Guide

4.1 Each test consists of eight replicates of the test material (for example, field-collected sediment or spiked sediment) and overlying water with five test organisms (recently-hatched tadpoles) per replicate. A laboratory control sediment (sometimes called a negative control) is used to provide *(1)* a measure of the acceptability of the test by indicating the quality of tadpoles, test conditions and handling procedures, and *(2)* a basis for interpreting data from other treatments. The test duration is ten days with an assessment of mortality and selected sublethal endpoints (that is, body width, body length) at the end of the test. Assessments of mortality can be made daily during the test and dead organisms removed. However, similar coloration of the tadpoles and sediment may make it difficult to see the organisms and sediment disturbance should be kept to a minimum. Alternative test durations and sublethal endpoints may be considered based on site-specific needs. The objective of the test is to evaluate whether test materials (spiked or field-collected sediments) are significantly more toxic than the laboratory control or reference sediment(s). Additional evaluations may be performed if an exposure gradient is tested. Statistical evaluations may be conducted to determine whether test materials are significantly more toxic than the laboratory control sediment or field-collected reference sample(s). If the test material is sediment spiked with a known concentration of a chemical stressor or if field-collected sediment contains a measured gradient of a particular chemical of concern, then point estimates (for example, median lethal concentrations (LC50s), 25 % inhibition concentrations (IC25s), or 50 % inhibition concentrations (IC50s)) may be calculated. Field-collected sediments often contain more than one potential chemical stressor and therefore calculating chemical-specific point estimates should only be done with caution. A reference-toxicant test should be run concurrently with a sediment test whenever a new batch or lot of organisms is used.

5. Significance and Use

5.1 While federal criteria and state standards exist that define acute and chronic "safe" levels in the water column, effects levels in the sediment are poorly defined and may be dependent upon numerous modifying factors. Even where USEPA recommended Water Quality Criteria (WQC, (**35**)) are not exceeded by water-borne concentrations, organisms that live in or near the sediment may still be adversely affected (**36**). Therefore, simply measuring the concentration of a chemical in the sediment or in the water is often insufficient to evaluate its actual environmental toxicity. Concentrations of contaminants in sediment may be much higher than concentrations in overlying water; this is especially true of hydrophobic organic compounds as well as inorganic ions that have a strong affinity for organic ligands and negatively-charged surfaces. Higher chemical concentrations in sediment do not, however, always translate to greater toxicity or bioaccumulation (**37**), although research also suggests that amending sediment with organic

TABLE 1 Advantages and Disadvantages for Use of Sediment Tests (Modified from Test Method E1706)

Advantages
Measure bioavailable fraction of contaminant(s).
Provide a direct measure of effects on sediment-associated receptors (benthos, larval amphibians), assuming no field adaptation or amelioration of effects.
Limited special equipment is required.
Methods are rapid and inexpensive.
Legal and scientific precedence exist for use; USEPA and ASTM standard methods and guides are available.
Measure unique information relative to chemical analyses or community analyses.
Tests with spiked chemicals provide data on cause-effect relationships.
Sediment-toxicity tests can be applied to all chemicals of concern.
Tests applied to field samples reflect cumulative effects of contaminants and contaminant interactions.
Toxicity tests are amenable to confirmation with natural populations (invertebrate or amphibian surveys).
Disadvantages
Sediment collection, handling, and storage may alter bioavailability.
Spiked sediment may not be representative of field contaminated sediment.
Natural geochemical characteristics of sediment may affect the response of test organisms.
Indigenous animals may be present in field-collected sediments.
Route of exposure may be uncertain and data generated in sediment toxicity tests may be difficult to interpret if factors controlling the bioavailability of contaminants in sediment are unknown.
Tests applied to field samples may not discriminate effects of individual chemicals.
Few comparisons have been made of methods or species.
Only a few chronic methods for measuring sublethal effects have been developed or extensively evaluated.
Laboratory tests have inherent limitations in predicting ecological effects.
Tests do not directly address human health effects.
Motile organisms may be able to avoid prolonged exposure to contaminated media so tests may overestimate actual exposure.
Species used in toxicity testing programs are typically chosen to be representative and protective of the organisms found on-site, but the use of surrogate species cannot precisely predict the health of ecological communities on-site.
Toxicity to organisms in situ may be dependent upon physical characteristics and equilibrium partitioning that are not readily replicated under laboratory conditions.

matter actually increases the bioaccumulation of contaminant particles (**38, 39**). Other factors that can potentially influence sediment bioaccumulation and toxicity include pH mineralogical composition, acid-volatile sulfide (AVS) and grain size (**40, 41**). Laboratory toxicity tests provide a direct and effective way to evaluate the effects of sediment contamination on environmental receptors while providing empirical consideration of all of the physical, chemical and biological parameters that may influence toxicity.

5.2 Amphibians are often a major ecosystem component of wetlands around the world, however limited data are available regarding the effects of sediment-bound contaminants to amphibians (**30-32, 41-43**). Laboratory studies such as the procedure described in this standard are one means of directly assessing sediment toxicity to amphibians in order to evaluate potential ecological risks in wetlands.

5.3 Results from sediment testing with this procedure may be useful in developing chemical-specific sediment screening values for amphibians.

5.4 Sediment toxicity test can be used to demonstrate the reaction of test organisms to the specific combination of physical and chemical characteristics in an environmental medium. The bioavailability of chemicals is dependent on a number of factors, which are both site-specific and medium-specific. Although many of these factors can be estimated using equilibrium partitioning techniques, it is difficult to account for all the physical and chemical properties which could potentially affect bioavailability. Sediment toxicity tests may be particularly applicable to evaluating hydrophobic compounds which may not readily partition into the water column. See Table 1 for a summary of advantages and disadvantages associated with sediment toxicity tests.

6. Interferences

6.1 *General Interferences:*

6.1.1 An interference is a characteristic of a sediment or a test system that can potentially affect test organism response aside from those related to sediment-associated contaminants. These interferences can potentially confound interpretation of test results in two ways: *(1)* toxicity is observed in the test sediment when contamination is low or there is more toxicity than expected, and *(2)* no toxicity is observed when contaminants are present at elevated concentrations or there is less toxicity than expected.

6.1.2 These general interferences may include: potential changes in contaminant bioavailability due to manipulation of field-collected sediments during collection, shipping, and storage; the influence of natural physico-chemical characteristics such as sediment texture, grain size, and organic carbon on the response of test organisms; tests conducted with field-collected samples usually cannot discriminate between effects of multiple contaminants. See Guide E1706 Section 6 for a detailed discussion of several general interferences that pertain to sediment toxicity testing.

6.1.3 Some interferences, such as the presence of indigenous organisms in field-collected sediments, may have less of an impact on toxicity tests conducted with larval amphibians than on tests conducted with sediment invertebrates.

6.2 *Species-Specific Interferences:*

6.2.1 Particular characteristics of individual species that were tested during the development of this method will probably not act as substantial interferences to completion of successful tests. Those species include *Rana pipiens*, *Bufo americanus*, *Rana clamitans*, *Rana palustris* (pickerel frog), *Rana sylvatica*, *Hyla chrysoscelis* (gray tree frog) and *Xenopus laevis*. However, because the sensitivity of these species to all potential sediment-associated contaminants is unknown, use of test organisms for which more toxicity data are available is recommended.

7. Facilities, Equipment, and Supplies

7.1 *Facilities*—While larval amphibians can be acclimated and held for short periods of time in static or static-renewal systems, continuous-renewal/flow-through conditions are preferable shortly after hatching. Tadpoles grow rapidly and, once feeding begins at about Gosner Stage 25 (**44**), ammonia concentrations are likely to increase and oxygen levels may be depressed, making flow-through conditions desirable. Culture/holding tanks and test chambers should be held at a constant temperature, either in an environmental chamber or temperature-controlled water bath. Addition of overlying water in a flow-through system should be gravity-fed from a water source that may be replaced via pumps. Overlying water should be near culture/test temperature although small temperature deviations should have little impact upon test water temperature at the slow rate of water replacement. Low dissolved oxygen concentrations may be remedied by increasing water replacement rates in small increments. If aeration is necessary, air should be free of contaminants including oil, dust and water; a filtration system may be desirable to remove bacterial contaminants. Lighting should be maintained at a 16-h light and 8-h dark cycle unless the test-specific protocol calls for an alternative photoperiod.

7.2 *Special Requirements*—Amphibian eggs and tadpoles can be highly sensitive to alterations in temperature, oxygen deprivation and handling. If eggs are received from an out-of-laboratory source, attention should be paid to how embryos are packed for shipment, shipment time and handling at the laboratory. Shipping containers should be durable, insulated and water tight. Embryos may be contained in large plastic bags sealed with rubber bands. Double bagging is recommended for added security. Oxygenation of the water containing the embryos is recommended before sealing the bags for shipment. Coolers containing embryos should be firmly taped shut before shipment. The use of ice packs or additional insulation in the shipping containers may be needed when outdoor temperatures are elevated or reduced. It is recommended that temperatures be monitored during shipment, if possible, or upon receipt at the laboratory. Upon receipt at the laboratory, eggs should be allowed to hatch with minimal disturbance.

7.3 *Equipment and Supplies*—All equipment used to prepare test sediments or reagents, transfer sediments or organisms and conduct tests, should be decontaminated as outlined below. Table 2 provides a list of the general equipment needed to conduct testing. Glass is the preferable material in which to conduct tests, however, alternative materials such as stainless steel, high-density polyethylene (HDPE), polycarbonate and fluorocarbon plastics may be appropriate, depending upon the contaminants of concern that might be present in the sediment. Used equipment should not be used if there is a possibility of residual contamination that cannot be removed via the washing process. In some cases, test substances present in field-collected sediments or introduced into spiked sediments may not be thoroughly washed from the test vessels. In these cases the test vessels should not be re-used. All new and used equipment needs to be washed in detergent and should be rinsed with dilute acid and deionized water. Rinsing with an organic solvent (for example, acetone) should also be considered for those materials that will not be damaged by the solvent (for example, some plastics) (see Test Method E1706 section 9.3.6 for a step-by-step cleaning procedure). Materials that should not contact overlying water include copper, cast iron, brass, lead, galvanized metal (that may contain zinc) and natural rubber.

TABLE 2 General Equipment Required for Conducting a 10-d Sediment Toxicity Test with *Rana pipiens*

Stainless steel bowls and spoons or auger to homogenize sediment
Testing chambers (usually 300 to 500 mL beaker with a small-mesh (300 µm) screen covering a hole drilled in the side of the beaker (secured with nontoxic silicone adhesive))
Transfer pipettes
Small nets
Dissecting microscopes
Dissolved oxygen meter and probe
Conductivity meter and probe
pH meter/selection ion meter and probe
Ammonia meter and probe
Reagents and equipment for hardness and alkalinity determinations
Temperature-controlled water bath or environmental chamber capable of controlling to 23 ± 1ºC
Flow-through water delivery system
Buffered 3-aminobenzoic acid ethyl ester, methanesulfonate salt (MS-222 anesthetic) solution.
Food source (TetraMin™)
Appropriate data forms
Metric ruler
Forceps
Statistical software

8. Test Material Collection and Processing

8.1 *Collection:*

8.1.1 Before field collection and preparation of sediments, a sampling/processing procedure should be established that outlines the site- or project-specific steps to be followed. The statistical analyses that will be applied to the data should be considered during the development of the sampling/processing procedure. See Guide E1391 for additional detail regarding methods for collecting, storing, and characterizing sediment samples.

8.1.2 Sediment should be collected with as little disturbance as possible. It may be desirable to collect sediments from a boat (even if wading is possible) to minimize sediment disruption.

8.1.3 Since the distribution of contaminants in sediment matrices can demonstrate a great deal of spatial variability (**45**), it is desirable to collect multiple replicates from within the delineated study area. At a minimum, multiple samples should be collected and thoroughly composited in the field so the sample better represents environmental conditions.

8.1.4 Large pieces of plant material and other debris, such as large rocks and glass, should be removed and discarded in the field. Alternatively, these materials can be removed in the laboratory prior to test setup.

8.1.5 In general, unless project specific conditions dictate otherwise, sediment should be collected from the top 15 cm of the native horizon, which generally represents the maximum bioactive zone and area of most probable exposure.

8.1.6 The exact collection procedures will depend upon study design. In deeper water where a boat is used, a benthic grab, dredge or corer should be used (Guide E1391). At locations where the water is very shallow, including saturated hydric soils, these devices can also be used or a clean trowel or shovel can be used. Whatever collection method is selected, all cleaning and decontamination protocols need to be followed to minimize sample contamination.

8.1.7 The testing procedure described in this standard requires a minimum of about one liter of sediment. Since this amount does not allow for accidental loss, spillage, analytical chemistry, or test reruns, collection of a minimum of two liters is recommended.

8.1.8 The most convenient sample containers are wide-mouth, high-density polyethylene (HDPE) bottles with a screw-on cap. Glass jars may be desirable for some studies where adsorption to plastic surfaces is of concern. However, glass containers require greater care in handling and packing for shipment and are generally more expensive than plastic jars.

8.2 *Storage:*

8.2.1 Light and heat can stimulate and accelerate chemical and biological reactions that may alter chemical composition, promote degradation of potential toxicants, and affect bioavailability. Samples, therefore, should be kept out of sunlight and stored in the dark under refrigeration. Samples should be cooled before shipping, unless the ambient temperature is already <10ºC. Target cooling temperature for sediments is about 4°C (Test Method E1367). Ice or blue ice should be included with the samples when they are shipped. Samples should not be frozen as freezing can alter sediment characteristics.

8.2.2 For additional information on sediment collection and shipment see Guide E1391.

8.2.3 It is desirable to initiate tests as soon as possible following field collection of sediments (Test Method E1706). Several studies have addressed the question of storage time for sediments, and the conclusions reached in these studies vary considerably. Where the potential chemical stressors are known to be recalcitrant, storage under the conditions described in 8.2.1 should allow the sample to remain stable for longer periods. However, some labile chemicals (for example, ammonia and volatile organics) can degrade or volatize during storage. For these labile materials, a maximum holding time of two weeks (from the time of sample collection to test initiation) is recommended **(46)**. However, more stable sediments can be stored for much longer periods of time with little change in toxicity.

8.2.4 During even short periods of storage, density differences will results in settling in samples, resulting in a heterogeneous mixture. Therefore, prior to test initiation, the sediment should be homogenized again, even if it was already mixed in the field. In most situations, overlying water should not be drained off the sample, but should be remixed with solid material. If, after 24 hours of undisturbed settling, >75 % of the sample volume can still be considered standing water, it may be desirable to remove some or all of that water so as to ensure that the test material will be a solid matrix.

8.3 *Manipulation:*

8.3.1 *Homogenization:*

8.3.1.1 Homogenization can be accomplished by using a tumbling or rolling mixer or other suitable apparatus. It can also be done using a stainless steel auger and drill or simply by hand with a stainless steel spoon. A minimum interval (at least three minutes) should be established for mixing each sample. A more heterogeneous sample would indicate the need for a longer mixing time. Additional large debris should be removed at this time. Sieving of samples is not recommended, however, indigenous organisms can be removed by hand during the mixing process. Special attention should be paid to any predaceous organisms that might be present in the collected sample. Augers, spoons, and any other equipment that comes in contact with the sediment during homogenization must be washed and decontaminated between samples.

8.3.2 *Sediment Spiking:*

8.3.2.1 Test sediment can be prepared by manipulating the properties of a control sediment (Test Method E1706). Mixing time **(45)** and aging **(47)** of spiked sediment can affect bioavailability of chemicals. If tests are initiated within only a few days of spiking a sediment, the spiked chemicals may not be at equilibrium with the sediment. There are not, however, specified equilibrium intervals for all chemicals that might be spiked into sediment. Such specifications would not be reasonable since sediment characteristics will play a major role in time to equilibration as well as equilibration concentrations. For a series of spiked sediment studies, where results will be compared, spiking methods should be consistent and the amount of time between spiking and test initiation should also be consistent.

8.3.2.2 The test material(s) should be at least reagent grade, unless a test using a formulated commercial product, technical-grade or use-grade material is specifically needed. Before a test is initiated, the following should be known about the test material (not all of this information may be available): *(1)* the identity and concentration of major ingredients and impurities, *(2)* solubility in test water and water used to prepare any stock solutions, *(3)* log K_{ow}, BCF for aquatic vertebrates (preferably amphibians), persistence in water and sediment, hydrolysis and photolysis rates, *(4)* estimated toxicity to the test organism, *(5)* toxicity to humans and potential handling hazards, *(6)* if and when analytical samples will be collected, how much material will be needed to obtain the needed resolution and preservation methods, and *(7)* recommended handling and disposal methods.

8.3.2.3 Different sediment spiking methods are available. Sediment spiking techniques used during development and validation of the amphibian sediment test method **(13)** were previously employed for incorporation of both inorganic contaminants and organic chemicals into sediment **(42)**. The procedure included: *(1)* place appropriate (considering testing and analytical needs) amount of sediment in a mixing jar, *(2)* if sediment is dry, wet it with deionized water to ensure holes in the sediment will remain open, *(3)* using a 10-mL or 5-mL pipet, punch at least five holes into the sediment to different depths, *(4)* distribute equally to each hole the volume of the

stock solution needed to achieve the desired target concentration of test material. The stock solution may be an inorganic salt dissolved in water (for example, copper as $CuCl_2$). If a hydrophobic chemical is to be tested, it may first be dissolved into a stock solution using a carrier solvent (for example, acetone or methanol). A surfactant should not be used in the preparation of a stock solution because it might affect the bioavailability, form or toxicity of the test material. If a carrier solvent is used, a solvent control must also be prepared which contains the solvent but not the contaminant to be tested. See USEPA (**1**), Guide E1391, and Test Method E1706 for additional details regarding sediment spiking techniques.

8.3.2.4 Once spiked, the sediments need to be thoroughly mixed to incorporate the chemical into the sediment and create a homogenized matrix. Homogenization methods include roller mixers, end-over-end mixers stainless steel kitchen mixers, mixing manually with a spoon or a combination of these. Mixing times, speeds and temperatures should be consistent among treatments, replicates and tests.

8.3.3 *Test Concentration(s) for Laboratory-Spiked Sediments:*

8.3.3.1 If a test is intended to generate an LC50, IC50 or IC25 of a test chemical, a concentration series should be created that will bracket that effect concentration. If mortality is one of the desired endpoints, at least one test concentration should produce greater than 50 % mortality and there should be two or more concentrations with partial mortality. Determining the concentration(s) that will result in desired lethal or sublethal effects can be difficult if *(1)* the environmental toxicity of the test material is unknown and/or *(2)* the impact(s) of sediment characteristics is/are unknown. The latter can be particularly important since there are many factors that can significantly affect toxicity (**37-41**). It may be desirable to conduct a range-finding test in which the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of ten. For example, test concentrations in a range-finding test may include the control, 10, 100 and 1000 mg/kg.

8.4 *Sediment Characterization:*

8.4.1 It is recommended that a subsample of each field-collected or spiked sediment be analyzed for at least the following parameters: pH, total organic carbon (TOC), particle size distribution (percent sand, silt, clay). Similar analyses should also be conducted on laboratory control sediment and reference sediment(s).

8.4.2 Further characterization may be warranted depending on the objectives of the study. This may include chemical analyses of inorganic and organic compounds of interest, ammonia, pore water chemistry, chemical oxygen demand, sediment oxygen demand, oxidation-reduction potential (Eh), acid volatile sulfides (AVS), and simultaneously extracted metals (SEM), or other analyses depending on the program.

8.4.3 Chemical and physical data should be obtained using appropriate standard methods whenever possible. For those measurements for which standard methods do not exist or are not sensitive enough, methods should be obtained from other reliable sources.

8.4.4 Sediment characterization helps to evaluate sediment homogenization and accuracy of sediment-spiking, and identifies potential chemical or physical stressors for test organisms.

9. Test Organisms

9.1 *Species*—Test organisms are recently hatched tadpoles of small North American anurans. The preferred species is the Northern Leopard Frog, *R. pipiens*. Sediment toxicity testing conducted with both *R. pipiens* and the American toad, *B. americanus*, during the development of this standard indicated that *R. pipiens* was generally more sensitive to spiked sediments containing metals (cadmium, copper, lead, or zinc) than was *B. americanus* (**13**). A review of amphibian data presented in U.S. EPA ambient water quality criteria documents for cadmium, copper, and zinc (**13**) and relative sensitivity data evaluating amphibian aquatic LC50s (**48**) indicate that *R. pipiens* is considered to be sensitive to metals, relative to other frog, toad, and salamander species. Other ranid species (*R. catesbeiana*, *R. palustris*) were also sensitive to the metals reviewed (**13, 48**). The potential for field-collection of *R. pipiens* eggs with minimal impact to local communities was also a consideration in the selection of this species as the preferred test species. Other species may be used for testing if handling and holding conditions are known.

9.2 *Sources*—While adults of several species of toads and frogs are available for most of the year from commercial suppliers of living organisms, availability of eggs is more limited. Eggs of *R. pipiens* can be collected in the wild during the spring. Since it may be difficult to distinguish between the eggs of related anuran species, collectors should be well-trained in species' habitats and identification. Collectors should comply with all state and federal regulations and be in possession of current collecting permits, if required. If possible, adult animals should also be collected for identification in the same area that eggs are being collected.

9.2.1 Eggs of *R. pipiens* can be obtained from commercial suppliers or be field collected from about November until April. Eggs that are produced and fertilized in the laboratory are preferable since the taxonomy is known. Researchers are encouraged to use available resources to find suppliers.

9.3 *Care and Handling*—Eggs received from commercial suppliers or collected in the wild should be subjected to a minimum of handling. Suppliers generally package and ship eggs in sealed bags or other containers that have been injected with oxygen (dissolved oxygen levels should be maintained above 4 mg/L to avoid stressing the test organisms). Hatching success is higher if handling of eggs is minimized; if possible eggs should left in the original shipping package until development is verified and organisms are near hatching stage. Upon receipt, bags containing eggs should be allowed to slowly rise (no more than 3°C per hour) to test temperature (avoid rapid temperature changes). If eggs arrive in containers that have not been injected with oxygen or otherwise cannot be left intact, organisms should be transferred to an aquarium or other holding container and slowly brought to test temperature.

9.3.1 Time to hatch will depend upon age at the time of shipping. Once the young embryos have developed into a recognizable tadpole and are actively moving, the bag can be

TABLE 3 Developmental Stages of Anuran Embryos (from Gosner (44) and Shumway (51))

Stage	Approximate Age at 18°C (h) for Stages 1 through 25	Major Characteristics/Formations of the Stage
1	0	Prior to fertilization
2	1	Appearance of post-fertilization gray crescent
3	3.5	Two blastomeres
4	4.5	Four blastomeres
5	5.7	Eight blastomeres
6	6.5	Sixteen blastomeres
7	7.5	Thirty-two blastomeres
8	16	Mid-cleavage
9	21	Late cleavage
10	26	Appearance of dorsal lip of blastopore
11	34	Mid-gastrula, blastoporal lip invaginating along semicircle
12	42	Late gastrula, blastoporal lip invaginating around the circular yolk plug. Yolk plug diameter ~ ⅛ diameter of gastrula
13	50	Neural plate, blastopore forming slit
14	62	Neural folds
15	67	Rotation of embryo
16	72	Neural tube
17	84	Tail Bud
18	96	"Tadpole" shape becoming distinct; muscular response to stimulation
19	118	Heart beat; external gill buds; hatching begins
20	140	Complete hatching; swimming upon physical stimulation; capillary circulation in first gill
21	162	Mouth open; transparent cornea; tail length approximately equal to length of head and body
22	192	Transparent epidermis; capillary circulation in tail; asymmetrical appearance from dorsal aspect; left gills filaments more apparent
23	216	Opercular fold apparent; asymmetrical from ventral aspect
24	240	Operculum covering right external gills; external gills on left side still apparent; sucker represented by two small prominences
25	284	Operculum complete; no external gill filaments; Sucker represented by two pigmented patches; begin feeding; gut clearly visible
26–30		Hind limb buds appear and grow progressively larger; spiracle present on left side (most North American tadpoles)
31		Toes begin to develop on hind limbs
32–37		Toes on hind limbs grow progressively distinct; all five toes apparent at stage 37
38–40		Toes continue to lengthen; metatarsal and subarticular tubercles develop
41		Tail begins to shorten; cloacal tail piece disappears; skin over forelimbs becomes transparent; lateral forelimb "bulges" appear
42–45		Forelimbs break through membrane; Face shortens; mouth lengthens; posterior edge of mouth extends beyond posterior edge of eye; tail absorption continues
46		Metamorphosis complete; tail stub usually present; froglets must have physical platform to leave the water

opened and the eggs/early stage tadpoles placed in an aquarium or other large chamber.

9.3.2 Once the eggs/tadpoles are released for the shipping container to an aquarium or other chamber, shipping water should be slowly replaced with culture/overlying water. This should be done by initially adding culture/overlying water at a proportion of no more than 10 % for one hour. If organisms do not appear to be adversely affected, increase the amount of culture/overlying water by about 15 to 25 %/ hour for 4 to 5 hours.

9.3.3 Additional acclimation of test organisms should not be needed under most circumstances.

9.3.4 Low dissolved oxygen will increase organism stress and may cause mortality in the holding chamber or result in increased mortality during a test. Dissolved oxygen should not be allowed to fall below 3.0 mg/L. If needed, gentle aeration should be initiated using a small pipette and low bubble rate.

9.3.5 Always wear laboratory gloves (for example, latex; talc-free) when handling eggs. Direct contact with eggs or tadpoles should be avoided to minimize stress on the organisms. Transfer eggs and tadpoles gently and with minimal handling time.

9.4 Once embryos have reached a distinctive tadpole shape (about Gosner stage 19-20) they are far less prone to mortality from handling.

9.5 A sub-sample of specimens should be collected and preserved for species verification.

10. Hazards

10.1 Some test materials, as well as some materials used to preserve test organisms, may be inherently hazardous. Caution needs to be used when handling these materials. Guidelines for the handling and disposal of hazardous materials should be strictly followed (Guide D4447). When working with any potentially hazardous materials, including those used for analytical measurements (for example, acid used during alkalinity titrations), users need to wear appropriate protective equipment (for example, safety glasses and gloves). Common laboratory protective wear should also be used to reduce exposure to potential biological hazards (for example *Salmonella*, *Vibrio* ssp.). All laboratory-specific health and safety considerations should be followed. (see Test Method E1706 for additional detail).

11. Procedure

11.1 *Experimental Design*—Each test consists of eight replicates of the test material (e.g., field-collected sediment or spiked sediment) and overlying water with five test organisms (recently-hatched tadpoles) per replicate. It may be necessary to make modifications of the basic experimental design to

accommodate project-specific circumstances, including shortage of available test sediment (for example,, scarce depositional areas in riverine systems), bioaccumulation (need for extra tissue) or additional analytical measurements. A laboratory control sediment (negative control) must be included with all tests and reference sediment(s) may be included when field-collected sediments are tested.

11.1.1 A laboratory control sediment is a sediment that is essentially free of contaminants and is used to ensure that contamination is not introduced during the experimental set up and that test organisms are healthy. This sediment is not necessarily collected near the site of concern. A reference sediment is collected near an area of concern and is used to assess sediment conditions exclusive of material(s) of interest. Testing a reference sediment provides a site-specific basis for evaluating toxicity.

11.2 *Initiating a Test:*

11.2.1 *Adding Sediment to Test Chambers*—The day before the test is to start (Day -1) sediment should be thoroughly homogenized and 100 mL of sediment is added to each test chamber. Overlying water (175 mL) is added to each test chamber in a manner that minimizes disturbance of the sediment. This can most easily be accomplished by pouring against the inside of the chamber. The sediment should be left undisturbed overnight.

11.2.1.1 On the day of test setup (Day 0), withdraw an adequate amount of overlying water from each treatment to conduct all necessary chemical characterizations and analyses. Removal of water should be done with as little sediment disturbance as possible. At a minimum, dissolved oxygen, temperature, pH, conductivity, hardness, alkalinity and ammonia should be measured in each treatment. If samples are collected for other parameters, such as metals, then proper handling and preservatives should be used (see Guide E1391 for additional detail).

11.2.1.2 Overlying water should be renewed during a test, unless nonrenewal is a fundamental part of the test design. Renewal may be done continuously through a water-delivery system, including diluters or drip-manifolds, or by static replacement. In either case, the volume of water addition in a 24-hour period should not exceed 2 to 3 volumes of overlying water (about 350 to 525 mL). A water-delivery system should be calibrated at test initiation and examined on a daily basis so all test chambers receive about the same amount of water. If manual water addition is conducted, no more than 80 % of the overlying water should be removed at any one time and sediment disturbance should be minimized. The toxicity test is designed to include both sediment and water column exposure to contaminants so it is important to maintain the indicated renewal rates in order to avoid excessive dilution of water column constituents that could lead to an underestimation of sediment toxicity.

11.2.2 *Addition of Test Organisms*—Test organisms should be handled as little as possible. Organisms should be added to the overlying water using a pipette with a large enough bore to prevent constriction and damage to the animals. The animals should be gently released just below the water's surface. The developmental stage (Gosner stage) of the tadpoles should be documented by examining a subset of at least 10 organisms.

11.2.2.1 Development stage should be determined in accordance with descriptions provided by Gosner (**44**). Table 3 provides a summary of the major characteristics of each stage between fertilization and metamorphosis.

11.3 *Monitoring a Test*—All chambers should be checked daily for dead organisms and behavior. Tadpole coloration often makes it difficult to see them against sediment, however, if dead organisms are found, they should be removed with a pipette. Animals that die during a test need only be kept if sublethal observations are to be made or tissue will be analyzed for chemicals of concern. Organisms need to be preserved appropriately for the analyses (see Guide E1688 for additional detail). The overlying water renewal system should be checked daily to ensure adequate flow and an acceptable addition rate. Screens on the outside of test chambers should be checked

TABLE 4 Test Conditions for Conducting a 10-d Sediment Toxicity Test with *Rana pipiens*

Parameter	Conditions
1. Test type:	Whole-sediment toxicity test with renewal of overlying water
2. Temperature:	23 ± 1°C
3. Light quality:	Wide-spectrum fluorescent lights
4. Illuminance:	About 100 to 1000 lux
5. Photoperiod:	16L:8D
6. Test chamber:	400 to 500-mL glass or plastic beaker or chamber with drainage system
7. Sediment volume:	100 mL
8. Overlying water volume:	175 mL
9. Renewal of overlying water:	Continuous flow-through of overlying water or daily static water addition (not to exceed 2 to 3 volume additions/day)
10. Age of organisms:	≤72 hours, 24 hours or less preferred at the start of the test
11. Number of organisms/chamber:	5
12. Number of replicate chambers/treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing (see 11.1)
13. Feeding:	4 mg of ground TetraMin™ per vessel daily after tadpoles reach stage 25; reduced proportionally with mortality
14. Aeration:	None, unless dissolved oxygen in overlying water drops below 3.0 mg/L.
15. Overlying water:	Site water, site water match (hardness and alkalinity), natural lake or groundwater, or reconstituted laboratory water (for example, U.S. EPA moderately hard (**5**))
16. Test chamber cleaning:	If screens become clogged during a test, gently brush the *outside* of the screen
17. Overlying water quality:	Hardness, alkalinity, conductivity, pH, dissolved oxygen, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily. Ammonia may also be measured periodically (Days 1, 3, and 7).
18. Test duration:	10 d
19. Endpoints:	Survival and growth
20. Test acceptability:	Minimum mean control survival of 80 %; mean body width of at least 4 mm and body length of at least 7 mm for test organisms in the control sediment. See Table 6 for additional performance-based criteria.

daily to ensure that water is adequately draining. Clogged screens can be brushed to remove impinged debris; cleaning and brushing should only be done with a small, clean brush, cleaning tool or gloved finger. Test conditions are summarized in Table 4 and a list of daily activities is presented in Table 5.

11.3.1 *Monitoring of Overlying Water Characteristics*—Conductivity, hardness, alkalinity, pH and dissolved oxygen must be measured in all treatments at the beginning and end of the test. Dissolved oxygen should also be measured daily. Temperature should be measured continuously in the environmental chamber or water bath and periodically in each treatment (for example, days 3, 6 and 9). If continuous temperature monitoring is not available then instantaneous temperature in each treatment should be measured daily. In any test chamber where mortality has occurred, dissolved oxygen and pH should be measured on the day when mortality was observed.

11.3.1.1 If dissolved oxygen in any one chamber of a treatment is less than 3.0 mg/L, then dissolved oxygen in other chambers within that treatment should be checked. The flow rate (drip rate if a continuous drip manifold is used) in any one chamber can be increased slightly to increase dissolved oxygen. All test chambers should be treated the same relative to test condition modifications (for example, increase in water delivery rate). If after one hour, dissolved oxygen is still <3.0 mg/L, then all of the test chambers within that treatment should be aerated. Set aeration tubes or pipettes so that the narrow tip is submerged not more than 0.5 cm. Bubble rate should be slow and should not disturb the sediment or overly agitate the water's surface to avoid the release of volatile substances. Occasional dissolved oxygen measurements of <3.0 mg/L during a test is not sufficient reason to discard test data, although evidence of extended oxygen depression should be considered with regard to possible adverse affects.

11.3.1.2 Ammonia should be measured in the overlying water on Day 0, at test termination and periodically during the test, for example, days 1, 3 and 7. If ammonia concentrations are >5.0 mg/L (NH_3-N) in any treatment, than a second sample should be collected and measured from another replicate. Tadpoles are sensitive to elevated ammonia, although *R. pipiens* has been found to be less sensitive than some other anurans (**7**, **49**). Elevated ammonia concentrations may be a reflection of sediment characteristics and should be taken into consideration when interpreting test results. Test specifications are listed in Table 4.

11.3.1.3 *Temperature*—Target test temperature is 23 ± 1ºC. Daily mean temperature (directly in the water bath or a surrogate test chamber in the water bath or environmental chamber) should be within 1ºC of 23ºC; instantaneous temperature should be 23 ± 3ºC. Continuous monitoring of bath or environmental chamber temperature is preferred.

11.3.2 *Feeding*—Feeding should begin when tadpoles reach Gosner stage 25 (**44**), that is, when an operculum develops and external gills disappear. About 3 to 4 mg of ground, dry TetraMin™ is added daily to each test chamber. Adding excess food should be avoided since it can cause dissolved oxygen depression and may also affect the toxicity of certain chemicals (**39**). Tadpoles in at least three chambers should be examined daily to determine if stage 25 has been reached (see Table 3 or (**44**)). Some toxicants may delay development; feeding of organisms may start on different days for different treatments. It takes about 3 to 5 days for newly-hatched tadpoles to reach stage 25. If older organisms were used, feeding will begin sooner. The amount of food added to each chamber should be decreased if some animals have died. In general, follow the USEPA (**34**) procedures for conducting short-term chronic tests with fathead minnows, *Pimephales promelas*. That is, if 50 % or more of the test organisms have died in a test chamber, reduce the amount of food by 50 %.

11.4 *Ending a Test*—Final water characterization measurements should be made and live organisms should be removed from each chamber with a pipette. All live organisms from a replicate chamber should be placed into a separate, small glass or plastic beaker or cup containing 10 to 20 mL of clean (unchlorinated) water (for example, USEPA Moderately Hard Water (see (**5**) or Guide E729)). All chambers should be carefully examined for any missing organisms. Dead tadpoles will decompose rapidly and may easily blend into sediment. Unaccounted-for organisms should be considered mortalities.

11.4.1 *Sublethal Measurements*—Live tadpoles should be anesthetized or euthanized before sublethal measurements are made. The use of a buffered 3-aminobenzoic acid ethyl ester

TABLE 5 General Activity Schedule for Conducting a 10-d Sediment Toxicity Test with *Rana pipiens*

Day	Activity
-1	Add homogenized sediment into each test chamber, place chambers into exposure system, and add overlying water.
0	Begin flow through system or conduct first water replacement if using static renewal. After at least one hour collect overlying water for initial water characterization (hardness, alkalinity, conductivity, pH, dissolved oxygen, and ammonia, and total residual chlorine). Add 5 tadpoles to each test chamber. Release organisms under the surface of the water. Archive and preserve 5 to 10 organisms for possible examination of metamorphic stage.
1 to 9	Measure temperature, dissolved oxygen. Measure ammonia periodically in each treatment during the toxicity test (for example, Days 1, 3, and 7). Observe behavior and metamorphic stage of test organisms. Remove dead organisms. Feed 4 mg of ground, dry TetraMin™ per chamber daily after tadpoles reach Gosner stage 25.
10	Measure temperature, dissolved oxygen, pH, conductivity. Collect samples for final water quality measurements (for example, hardness, alkalinity, ammonia), as indicated in project requirements. Remove and count live organisms from each test chamber and transfer them to small beakers (glass or plastic) containing 10 to 20 mL of clean (unchlorinated) water. Euthanize or anesthetize test organisms prior to making sublethal measurements. Measure the maximum body width and body length (snout-to-vent length).

(MS-222) solution is recommended. To each of the small beakers or cups containing live tadpoles, add about 1 mL of a MS-222 stock solution (2 g/L) buffered to about pH 7 using an appropriate buffer medium (for example, sodium bicarbonate). If organisms continue to move after several minutes, add a few more drops of the MS-222. Tadpoles should not be left for an extended period of time in the MS-222 solution as it may cause disintegration of tissue.

11.4.1.1 Using a metric ruler, measure the maximum body length along the center line of the body, excluding the tail (snout–to-vent length). Also, measure the maximum body width. Do not push down on the tadpole body as that will distort these measurements.

11.4.2 Digital photographs and digitizing software may also be used to quantify sublethal measurements.

11.4.3 Statistical evaluations for lethal and sublethal endpoints may be conducted using comparisons to results from the laboratory control or a field-collected reference sample(s). If the test was one in which sediment was spiked with a hydrophobic test material dissolved in a solvent carrier and a solvent control was included in addition to a laboratory control sediment, then survival and growth should be compared between the two controls. If a statistically significant difference is detected between the controls, then only the solvent control may be used for meeting the acceptability of the test and as the basis for calculation of results. The laboratory control may provide additional information on the general health of the test organisms. If no statistically significant difference is detected between the controls, the data from both controls may be pooled and used as a basis for meeting acceptability criteria and as a basis for calculation of results. If the solvent control is markedly different from the laboratory control, it is possible that the data are compromised by experimental artifacts and may not accurately reflect the toxicity of the test material in natural sediments. In such circumstances, the test may need to be repeated or alternative means of test material introduction explored. A discussion of possible statistical evaluations is presented in Appendix X2 but may be modified based on project-specific requirements.

11.5 *Studies Conducted Beyond Ten Days*—If site-specific information indicates that longer duration toxicity tests should be conducted, the daily activities described previously should be followed until test termination.

11.5.1 Activities conducted at test termination will be similar to those conducted for the 10-d toxicity test but may also include inspection for deformities, observations of impaired behavior (prior to anesthetizing), or developmental stage. Feeding should be increased in proportion to the increase in body size of the test organisms. If growth is not affected, the amount of food can be increased by about 2 mg per chamber every five days; not to exceed 12 mg per chamber. If the growth of organisms is diminished, feeding levels should remain unchanged or be increased at a slower rate. Excess food on the surface, sediment or sides of the test chambers indicates that too much is being added and the amount of food should be reduced. At metamorphosis, most larval anurans stop eating as their internal and external physiology undergoes substantial alterations in the shift from a fully aquatic tadpole to an amphibious adult (**43**). As the organisms within a replicate approach late-stage metamorphosis, the amount of food consumed will drop substantially and feeding amounts should proportionally decrease to initial levels or less. At some point, if no feeding behavior is observed and unconsumed food is present, feeding may be stopped within a particular replicate.

11.5.2 If the toxicity test is to be conducted through metamorphosis, some modifications would need to be made in the test system. At complete metamorphosis (about Gosner stages 45 and 46) froglets crawl out of the water. Failure to provide a means of leaving the water will result in tadpole death. Providing an "emergence platform" may be difficult if the original test chambers were beakers or similar vessels. Sediment, water and organisms can be transferred to a vessel with a larger surface area that provides better access for the researcher (for example, a 12 by 25 cm plastic chamber). The emergence platform can be constructed in several ways, but the froglet will need to be able to crawl from the water to air. Possible emergence structures include inclined glass or plexiglass, bricks or stones, sponges and arched pieces of heavy, nylon netting. Any material used as an emergence structure needs to be decontaminated as outlined in 7.3 and should not block water circulation or prevent tadpoles from moving freely about the test chamber.

11.6 *Reference Toxicant Testing*—Reference toxicant tests involve exposing organisms that are used to start a sediment study to known concentrations of a specific reagent-grade reference chemical in water-only exposures in order to assess their sensitivity to a toxicant challenge. Organisms of a given species should demonstrate a consistent response to a reference toxicant. Deviations outside an established normal range may indicate a change in the sensitivity of the test organism. A reference toxicant test must be conducted with each new lot or batch of test organisms that are used to initiate a test. Test conditions for conducting reference-toxicity tests with *R. pipiens* are outlined in Table 6. The procedures can also be used for conducting reference-toxicity tests with the test organisms outlined in Appendix X1.

11.6.1 There are several chemicals that are used as reference toxicants. Copper chloride ($CuCl_2$) has been found to produce consistent responses from the test organisms when organism age and test water are held constant. Other possible reference toxicants include salts such as NaCl and KCl. A reference-toxicant concentration series should be selected that will provide partial mortalities at two or more concentrations of the test chemical in order to allow calculation of appropriate point estimates (for example, LC50, EC50).

11.6.2 A reference toxicant control chart should be prepared for each toxicant (if difference ones are used) that progressively illustrates reference toxicant test results. Results should be illustrated as the calculated value for a test, bracketed by the upper and lower control limits. The control chart should include the 20 most recent reference toxicant data points (**34**).

11.6.3 If the reference toxicity results from a given study fall outside the "expected" range (more than 2 standard deviations), the sensitivity of the organisms and the acceptability of the study may be in question. However, at a 0.05 probability level, it is expected that, by chance alone, one in 20

TABLE 6 Recommended Test Conditions for Conducting Reference-Toxicity Tests

Parameter	Conditions
1. Test type:	Water-only test
2. Dilution series:	Control and at least 5 test concentrations (0.5 dilution factor)
3. Toxicant:	KCl, NaCl, or $CuCl_2$
4. Temperature:	23 ± 1°C
5. Light quality:	Wide-spectrum fluorescent lights
6. Illuminance:	About 100 to 1000 lux
7. Photoperiod:	16L:8D
8. Renewal of water:	At least every 48 hours
9. Age of organisms:	≤72 hours, ≤24 hours preferred
10. Test chamber:	250-500 mL glass or plastic beaker
11. Volume of water:	100 mL (minimum)
12. Number of organisms/chamber:	5
13. Number of replicate chambers/treatment:	3 minimum
14. Feeding:	4 mg/day to each test chamber when organisms reach Gosner stage 25
15. Substrate:	None
16. Aeration:	None, unless DO ≤ 3 mg/L
17. Dilution water:	Culture water, well water, surface water, site water, or reconstituted laboratory water (for example, USEPA moderately hard **(5)**)
18. Test chamber cleaning:	None
19. Water quality:	Hardness, alkalinity, conductivity, dissolved oxygen, and pH at the beginning and end of a test. Temperature daily.
20. Test duration:	7 d
21. Endpoint:	Survival (LC50) and growth (IC25)
22. Test acceptability:	80 % control survival

TABLE 7 Test Acceptability Requirements for a 10-d Sediment Toxicity Test with *Rana pipiens*

A. It is recommended for conducting a 10-d test with *Rana pipiens* that the following performance criteria be met:
1. Age of *R. pipiens* at the start of the test must be ≤72 hours.
2. Average survival of *R. pipiens* in the control sediment must be greater than or equal to 80 % at the end of the test. Growth of test organisms should be measurable in the control sediment at the end of the 10-d test (mean body width of at least 4 mm and body length of at least 7 mm for test organisms in the control sediment).
3. Hardness, alkalinity, and ammonia of overlying water typically should not vary by more than 50 % during the test, and dissolved oxygen should be maintained above 3.0 mg/L in the overlying water.
B. Performance-based criteria for maintaining *R. pipiens* include the following:
1. It may be desirable for laboratories to periodically perform water-only reference toxicity tests to assess the sensitivity of culture organisms (see 11.6). Data from these reference toxicity tests could be used to assess genetic strain or life-stage sensitivity of test organisms to select chemicals.
2. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.
3. Physiological measurements such as lipid content might provide useful information regarding the health of the cultures.
C. Additional requirements:
1. All organisms in a test must be from the same source.
2. Storage of sediments collected from the field should follow guidance outlined in 8.2.
3. All test chambers should be identical and should contain the same amount of sediment and overlying water.
4. Negative-control sediment and appropriate solvent controls must be included in a test. The concentration of solvent used must not adversely affect test organisms.
5. Test organisms must be cultured and tested at 23°C (±1°C).
6. The daily mean test temperature must be within ±1°C of 23°C. The instantaneous temperature must always be within ±3°C of 23°C.
7. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

test results would fall outside the control limits. If more than one in 20 reference toxicant tests fall outside of the control limits, the laboratory should investigate possible sources of variability and take corrective action, if appropriate. If serious problems are not found, then associated test results may be considered acceptable.

12. Acceptability of Test

12.1 Acceptable survival in the test control is 80 % or greater. Control organisms (*R. pipiens*) should also have a mean body width of at least 4 mm and a body length (snout-to-vent) of 7 mm. If alternative test species are used , researchers may need to complete appropriate data gathering tests to determine acceptable size criteria prior to conducting the toxicity tests. If control performance does not meet these criteria, then the test data should be examined to determine if it is acceptable. Test acceptability criteria are presented in Table 7. Even if control performance does not meet these criteria, test data may still be valuable and yield important results. The following test data should be examined:

12.1.1 Survival in all test treatments. If survival in all test treatments is greater than in the control, then statistical evaluations of test sediments against the laboratory control do not need to be conducted. Statistical comparisons against the reference sediments may still be conducted.

12.1.1.1 If poor performance is observed in the laboratory control, such studies should be repeated to ensure accurate results. However, the scope or sampling associated with some studies may make it difficult or impossible to repeat a study. There may be cases where performance in the negative control is poor, but performance criteria are met in reference sediment included in the study design. In these cases, it might be reasonable to infer that other samples that demonstrate organism performance equivalent to, or better than, the reference sediment are probably not toxic; however, any samples showing poor performance should not be judged to be toxic, since it

is unknown whether the factors that caused poor control performance might have also caused poor performance in the test treatments.

12.1.2 Variability within a treatment. If mortality is highly variable and scattered throughout the test, then the test might not be acceptable. Highly variable survival may be due to variations in water chemistry (for example, low dissolved oxygen or elevated ammonia due to excess food in some chambers), variability in organism health, or differences in how chambers were treated (for example, different amounts of food or flow rates of overlying water).

12.2 There are no specific acceptability requirements for survival in test treatments collected from reference stations. If reference sediment was collected and if survival in the reference sediments is significantly reduced, then questions are raised as to the appropriateness of the reference site.

12.3 Reference toxicant data for a given batch of organisms should fall within the historical 95 % limits for that species. However, data falling outside the range does not necessarily indicate automatic rejection of the data.

13. Report

13.1 Report the following information:

13.1.1 Identity of the test material (for example, test sediments and reference sediment, if collected), investigator(s) name, location of laboratory, and dates of test initiation and termination.

13.1.2 Source of test material (if a specific chemical or compound), its lot number, composition (identities and purity), known physical and chemical properties and the identity and source of any solvent used.

13.1.3 Source of the laboratory control sediment and overlying water.

13.1.4 Chemical characteristics of test material, laboratory control sediment, and overlying water, if available.

13.1.5 Source of test organisms, scientific name (and subspecies, if appropriate), life stage, treatments, acclimation procedures and food.

13.1.6 Description of the experimental design, test chambers or compartments, amount of sediment and overlying water, replicates, organisms per replicate, lighting, food type and feeding rate.

13.1.7 Range of measured concentrations of dissolved oxygen, temperature, pH and conductivity of overlying water.

13.1.8 Chemical and biological monitoring information recorded on daily data sheets during the toxicity test.

13.1.9 A table that lists the percent mortality and mean sublethal results (that is, body width, body length) for each test material.

13.1.10 The names of the statistical tests employed, the alpha-levels of the tests, and some measure of the variability of the hypothesis tested.

13.1.11 Anything unusual about the test and any deviations from the test-specific protocol or procedures followed.

14. Precision and Bias

14.1 *Determining Precision and Bias*—Precision is a term that describes the degree to which data generated from replicate measurements differ and reflects the closeness of agreement between randomly selected test results. Bias is the difference between the value of the measured data and the true value and is the closeness of agreement between an observed value and an accepted reference value (Practices E177 and E691). Quantitative determination of precision and bias in sediment testing of aquatic organisms is difficult or may be impossible in some cases, as compared to analytical (chemical) determinations. This is due, in part, to the many unknown variables which affect organism response. For a detailed discussion of precision as it relates to sediment toxicity testing, see Section 17 in Test Method E1706.

14.1.1 *Bias*—The bias of toxicity tests cannot be determined since there is no acceptable reference material. The bias of the reference-toxicity tests can only be evaluated by comparing test responses to control charts be evaluated by comparing test responses to control charts. For a detailed discussion of bias as it relates to sediment toxicity testing, see Section 17 in Test Method E1706.

14.1.2 The sensitivity of a toxicity test will depend upon the number of replicates per concentration or treatment, the variability within that treatment (among replicates), the probability levels (alpha and beta) and the statistical test used. Tests with anuran larvae have demonstrated that variability may occur within a treatment. This is especially the case for sublethal growth parameters where particularly small or large organisms can occur within a single treatment. Such differences in size may represent natural physiological differences (that is, poor health) or behavioral differences in individuals that affect access and consumption to available food and subsequent lower growth rates. The presence of unusually small or large specimens within a replicate chamber is to be occasionally expected and is not reason to discard individual measurements as outliers, unless all or most individuals in a single replicate exhibit mortality or growth patterns that are substantially different from other replicates within a treatment. Such a situation may indicate poorly homogenized sediment, technician error at test initiation or the presence of a highly-consolidated particle containing a toxic substance that is not representative of the sediment as a whole. In such cases, an outlier test may be appropriate to determine whether the replicate should be excluded from analysis. Exclusion of replicates should be avoided, however and every effort should be made collect enough sediment for a full eight replicates, in order to increase the statistical power of the test and reduce the effects of replicate variability **(50)**.

14.1.3 Intralaboratory precision data are routinely calculated for test organisms using water-only exposures to a reference toxicant, such as NaCl or KCl (as described in 11.6). Intralaboratory precision data should be tracked using a control chart. For reference toxicant tests with anurans, both survival and growth parameters should be tracked. Reference toxicant tests should be of a sufficient duration to achieve measurable growth (relative to the size of organisms at test initiation). For anurans, a minimum of seven days is recommended. Each laboratory's reference-toxicant data will reflect conditions unique to that facility, including dilution water, culturing, and other variables. The conditions for the reference toxicant test,

such as water type, test containers, organism age, feeding and concentration series, should remain the same. Altering test variables will introduce variation, wider confidence intervals and will compromise the integrity and usability of the reference toxicant data as a means of tracking intralaboratory precision.

14.1.4 Before conducting tests with potentially contaminated sediment, it is strongly recommended that the laboratory conduct the tests with control sediment(s) alone. Results of these preliminary studies should be used to determine if the use of the control sediment and other test conditions (that is, water quality) result in acceptable performance in the tests. If organism performance in the selected control sediment is inconsistent, an alternative sediment should be selected.

15. Keywords

15.1 amphibian; bioavailability; *Bufo* spp.; hydric soils; *Rana* spp.; *Rana pipiens*; sediment; toxicity; wetland

APPENDIXES

(Nonmandatory Information)

X1. LIST OF ALTERNATIVE SPECIES

X1.1 *Use of Alternative Species*—Although this procedure was developed with *R. pipiens*, it might be necessary to use alternative species when required by regulation or limited by seasonal availability of test organisms. Deviations from the procedures outlined in Table 4 should be recorded and it may be difficult to compare data between toxicity tests conducted with *R. pipiens* and alternative species.

X1.2 *Recommended Anurans*—Other members of the family Ranidae (for example, *R. sphenocephala*, *R. palustris*, or *R. catesbiena*) and Bufonidae (for example, *B. americanus* or *B. fowleri*) might be best suited for conducting a whole-sediment exposure toxicity test due to the commercial availability of eggs. High egg production, relevant geographical range, short hatching periods, and sensitivity to contaminants should be considered in selecting alternative species. *Xenopus laevis* may be considered as an alternative species due to the generally consistent availability of eggs; however, researchers should review existing data on the relative sensitivity to some contaminants (**48**).

X1.2.1 Standard E1439-98 includes a methodology for exposing *X. laevis* to whole sediments (referred to as solid phase sample testing). This methodology is an alternative to FETAX studies conducted in aqueous solutions. Although *Xenopus* is not native to the United States, the standardized, FETAX testing protocol, the availability of test organisms, and ease of use of *Xenopus* in the laboratory has made it a popular test species for amphibian toxicity testing.

X1.2.1.1 The FETAX solid phase testing may be performed in 250 mL specimen bottles or similar capped vessels equipped with a 55 mL glass tube with Teflon mesh insert as the exposure chamber. For screening tests, 35 g of sediment (dry weight) should be placed in the bottom of the vessel, with the Teflon mesh insert added, and should be filled with 140 mL of FETAX Solution. Blastulae stage embryos are placed directly on the mesh insert that rests directly over the top of the soil or sediment in the sediment/water interface region. Four to six dilutions ranging from 0 to 100 % soil sample and a FETAX Solution control are typically tested. Each sample should be tested in triplicate. Solutions and soils or sediments should be changed every 24 hours of the four-day test. At the end of the four-day exposure period, surviving embryos should be preserved in 3 % (w/v) formalin (pH 7.0) and morphological characteristics evaluated using a dissecting scope. Growth may be determined using a digitizing software package.

X1.2.1.2 While the alternative FETAX methodology exposes young amphibians to sediments there are several differences relative to the test conditions presented in Table 4. Primarily these differences are related to test duration and the age of the test organisms. The FETAX test is a rapid test designed to identify developmental toxicants. It is conducted over a relatively short duration (4 d) with recently fertilized embryos (mid blastula to early gastrula) and evaluates malformations, in addition to mortality and growth. The test conditions presented in Table 4 indicate a longer test duration (10 d) with older test organisms (≤72 hours old). This methodology evaluates survival and growth of tadpoles exposed directly to sediment and overlying water. The FETAX methodology is conducted with an amphibian species that is not native to North America. Although *X. laevis* may be available with less seasonal variability, in some cases it may be preferable to conduct a toxicity test with a species that is native to the test site.

X2. DATA ANALYSIS

X2.1 *General*—Test Method E1706 provides guidance on data analysis. The following sections briefly summarize this guidance. Mortality or apparent size reduction in any sediment treatment is not necessarily an indication of toxicity. Statistical analysis is used to determine if apparent differences are significant (**52-54**). Organism response to test sediments is typically compared to the control response. If a reference sediment (for example, upstream or independent of a study site) is also collected, then test sediment results may be statistically compared against the reference sediment. Two types of data are obtained from the toxicity test: acute (mortality) and chronic (width and length). Each data type should be analyzed independently. If other measurements are also obtained (for example, weight or tissue burden) then those data can also be analyzed separately.

X2.2 *Forms of Evaluation*—Data analysis is in two general forms: hypothesis testing and point estimation. Hypothesis testing involves assigning an alpha level for the analysis and then, using that criterion, determining which treatments are significantly different from the control. If only field-collected sediment is tested, then data analysis will typically consist only of hypothesis testing. If however, a series of sediment dilutions were prepared (that is, mixing test sediment with control sediment at fixed percentages [6.25, 12.5, 25, 50]), or if spiked-sediment samples are prepared representing a true concentration gradient for chemical(s) of concern, then point estimates can be made. A point estimate, such as an LC50, is a concentration of test media at which a certain effect (for example, half the test organisms die) is determined to occur. General guidance for conducting these analyses is given in the following sections.

X2.2.1 *Hypothesis Testing*—Hypothesis testing should follow the same general structure as described by Test Method E1706 and by U.S. Environmental Protection Agency (**1, 34**). In summary, mortality/survival data are analyzed first. If there is a significant reduction in survival in any treatment, that treatment is dropped from analysis of sublethal data. Determination of significant effects is dependent upon the predetermined alpha level. The alpha level, or α, is defined as the probability of committing a Type I statistical error—rejecting the null hypothesis (H_o) of no effect, even if H_o is true. That is, concluding a sample is toxic, even when it isn't (Table X2.1).

X2.2.1.1 The majority of studies in environmental toxicology are analyzed with an α of 0.05, which means there is a theoretical 5 % chance that a Type I error will be committed. The α level is not fixed and can be changed, depending upon the objectives of the study. A lower α—0.01 for example—will reduce the likelihood of a Type I error. However, it will also increase the likelihood of a Type II error (β), that is, concluding that a sample is not toxic when it, in fact, is. Historically, β and its inverse (1-β), which is the associated power of the test, have generally been ignored by environmental researchers. However, because the power of a test is defined as the probability of correctly detecting a true toxic effect, considering β may be important in designing a study. If α is held constant, for example, β decreases (and test power increases) as the sample size increases and variance decreases (**50**).

TABLE X2.1 Statistical Errors

Decision	If H_o is True	If H_o is False
H_o Rejected	Type I error (α)	No error
H_o Accepted	No error	Type II error (β)

X2.2.1.2 Since survival data often demonstrate non-normal distributions, proportional survival data are first transformed using an arc sine-squareroot transformation. The normality and homogeneity of variance are then evaluated using tests such as Shapiro-Wilk's and Bartlett's, respectively. If data are found to meet the normality and homogeneity of variance requirements of parametric tests, then differences from the control can be analyzed with Dunnett's Procedure (for an equal number of replicates) or a T-Test with Bonferroni adjustments (for unequal replicates). If data do not meet the assumptions for a parametric test, then nonparametric (rank) tests have to be used. The most common tests are Steel's Many-One Rank Test (for equal replicates) or Wilcoxon Rank Sum Test with Bonferroni adjustments (for unequal replicates).

X2.2.1.3 While these statistical tests are the ones most commonly used in the analysis of toxicity data, they are not the only ones available. For example, the objective of the study may be to determine if test sediments are significantly different from each other, as well as from the control. In that case, analysis of variance with Tukey's multiple range test (parametric) or a Kruskal-Wallis test (nonparametric) may be appropriate. Because of the many tests that are available, it is important that the project goals be thoroughly defined before data are collected.

X2.2.1.4 Sublethal effects are analyzed after mortality effects have been evaluated. Individual sublethal measurements are averaged to produce a mean width and length (per surviving organism) for each replicate. If there was significant mortality in any test treatment, that treatment is typically dropped from analysis of sublethal effects. Sublethal measurements are continuous data and therefore do not need to be transformed (arc sine-squareroot) before analysis. With that exception, the analysis of sublethal endpoints is the same as for survival.

X2.2.2 *Point Estimates*—Point estimations for individual chemicals of concern are seldom used in sediment tests conducted with field-collected samples because there is generally not a single concentration gradient for the particular chemical of concern. In addition, field-collected sediments may contain multiple toxicants that could act independently or have synergistic, additive, or antagonistic effects. For example, if a sediment (for example, from a historical mining district) has high concentrations of copper, zinc, and cadmium, all of which may be at toxic levels, a point estimate based on the concentration of any one metal may be meaningless because of the presence of the other metals. However, point estimates could be calculated based upon the percent (weight or volume) of a

test sediment mixed with a nontoxic control sediment. If this method is used, then both sediments should have about the same moisture fraction so that the percentage estimates are reasonably accurate. Point estimates could also be used if samples are collected along a known concentration gradient for one particular chemical and no other chemicals of concern are present. Finally, if spiked sediment tests are conducted where different treatments of sediment contain variable but known quantities of a particular chemical, then point estimates can be made.

X2.2.2.1 Any of the point estimation procedures calculate a concentration (mass per volume or percent) at which a certain effect will occur. An LC50, for example, is the concentration at which 50 % of the organisms are expected to die while an IC25 is the concentration which causes a 25 % reduction in the endpoint of interest. The manner in which LC50s or other point estimates are calculated varies with the structure of the data. For example, if the responses in the test treatments are all or nothing (either everything is alive or everything is dead), than the simplest method—graphical—is used. LC50s using the graphical method, like the name implies, are calculated on graph paper, although a simpler method is simply calculating the geometric mean of the highest "all-alive" concentration and the lowest "all-dead" concentration. If there is partial mortality in any test treatment then a Spearman-Karber, Trimmed Spearman-Karber, or Probit method should be used. These methods are described in detail by U.S. Environmental Protection Agency (**55**). In brief, if there are two or more treatments with partial mortality, then use of the Probit method (parametric) is indicated. In situations where the Probit method is inappropriate due to non-normal or significantly heterogeneous data, the Trimmed Spearman-Karber or Spearman-Karber Methods may be used. These LC50 procedures are available with a variety of computer software programs (**52-54**).

X2.2.2.2 LC50 models, by definition, are used to calculate point estimates for mortality endpoints, although the models can also be used to calculate point estimates for nonlethal endpoints (for example, median effects concentrations (EC50s)). The Linear Interpolation Method was developed for the general application to data generated during chronic toxicity tests. The endpoint generated by the Linear Interpolation Method is an ICp value, where IC = Inhibition Concentration and p is the percent effect. The value of p can be adjusted, although the most typical values are 25 and 50. The Linear Interpolation Model assumes a linear response from one concentration to the next and assumes that the mean response of the next higher concentration will be equal to or less than the preceding concentration. If this is not the case, the data are adjusted by smoothing. A more thorough discussion of the Linear Interpolation Model is provided by Norberg-King (**56**).

REFERENCES

(1) U.S. Environmental Protection Agency, "Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates–Second Edition," EPA/600/R-99/064, 2000.

(2) U.S. Environmental Protection Agency–U.S. Army Corps of Engineers, "Evaluation of Dredged Material Proposed for Ocean Disposal–Testing Manual," EPA 503/8-91/001, 1991.

(3) U.S. Environmental Protection Agency–U.S. Army Corps of Engineers, "Evaluation of Dredged Material Proposed for Discharge in Waters of the U.S.–Testing Manual," EPA 823/B-98/004, 1998.

(4) Francis, P. C., Birge, W. J., and Black, J. A., "Effects of Cadmium-Enriched Sediment on Fish and Amphibian Embryo-Larval Stages," *Ecotoxicology and Environmental Safety*, Vol 8, 1984, pp. 378–387.

(5) Berrill, M., Coulson, D., McGillivray, L., and Pauli, B., "Toxicity of Endosulfan to Aquatic Species of Anuran Amphibians," *Environmental Toxicology and Chemistry*, Vol 17, 1998, pp. 1738–1744.

(6) Edginton, A. N., Stephenson, G. R., Sheridan, P. M., Thompson, D. G., and Boermans, H. J., "Effect of pH and Release® on Two Life Stages of Four Anuran Amphibians," *Environmental Toxicology and Chemistry*, Vol 22, 2003, pp. 2673–2678.

(7) Jofre, M. B. and Karasov, W. H., "Direct Effect of Ammonia on Three Species of North American Anuran Amphibians," *Environmental Toxicology and Chemistry*, Vol 18, 1999, pp. 1806–1812.

(8) Snodgrass, J. W., Hopkins, W. A., Jackson, B. P., Baionno, J. A., and Broughton, J., "Influence of Larval Period on Responses of Overwintering Green Frog (*Rana clamitans*) Larvae Exposed to Contaminated Sediments," *Environmental Toxicology and Chemistry*, Vol 24, 2005, pp. 1508–1514.

(9) Leney, J. L., Drouillard, K. G., and Haffner, G. D., "Does Metamorphosis Increase the Susceptibility of Frogs to Highly Hydrophobic Contaminants?" *Environmental Science and Technology*, Vol 40, 2006, pp. 1491–1496.

(10) Ankley, G. T., Tietge, J. E., DeFoe, D. L., Jensen, K. M., Holcombe, G. W., Durhan, E. J., and Diamond, S. A., "Effects of Ultraviolet Light and Methoprene on Survival and Development of *Rana pipiens*," *Environmental Toxicology and Chemistry*, Vol 17, 1998, pp. 2530–2542.

(11) James, S. M. and Little, E. E., "The Effects of Chronic Cadmium Exposure on American Toad (*Bufo americanus*) Tadpoles," *Environmental Toxicology and Chemistry*, Vol 22, 2003, pp. 377–380.

(12) U.S. Environmental Protection Agency, "Method for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod *Leptocheirus plumulosus*," EPA/600/R-01/020, 2001.

(13) Naval Facilities Engineering Command (NAVFAC), "Development of a Standardized Approach for Assessing Potential Risks to Amphibians Exposed to Sediments and Hydric Soils," March, 2004 .

(14) Naval Facilities Engineering Command (NAVFAC), "Laboratory Validation Plan for ESTCP Project CU-0514 Demonstration and Certification of Amphibian Ecological Risk Assessment Protocol," December 2005.

(15) Loveridge, A. R., Lavoie, D. R., Corl, W. E., Jackson, R., Speicher, J., Pillard, D. A., and Christopher, J., "Use of a New Standardized Test for Assessing Potential Risks to Amphibians Exposed to Whole Sediments," Poster presented at Annual Meeting of Society of Environmental Toxicology and Chemistry, Portland, OR. November 2004.

(16) Environmental Chemical Corporation, "Draft Ecological Risk Assessment Addendum–Area of Concern CS-10 Detail F, Massachusetts Military Reservations, Cape Cod, MA," October, 2004.

(17) ENSR, "RAM Completion Statement & RAO Class A-2 Statement, Wetlands Reclamation Project Adjacent to MassHighway Maintenance Depot, Marrett Road (Rte. 2A) Lexington, MA MADEP RTN: 3–20801," November, 2002.

(18) ENSR, "Draft Baseline Ecological Risk Assessment, Sixty-One Industrial Park Site Memphis, Tennessee," May, 2007.

(19) Lande, S. P. and Guttman, S. I., "The Effects of Copper Sulfate on the Growth and Mortality Rate of *Rana pipiens* Tadpoles," *Herpetologia*, Vol 29, 1973, pp. 22–27.

(20) Birge W. A. and Black, J. A., "Effects of Copper on Embryonic and Juvenile Stages of Aquatic Animals, " J. O. Nriago (ed.), *Copper in the Environment*, 1979, pp. 373–399.

(21) Khangarot, B. S., Sehgal, A., and Bhasin, M. K., "Man and Biosphere–Studies on the Sikkim Himalayas. Part 5: Acute Toxicity of Selected Heavy Metals on the Tadpoles of *Rana hexadactyla*," *Acta Hydrochimica et Hydrobiologica*, Vol 13, 1985, pp. 259–263.

(22) Mudgall, C. F. and Patil, S. S., "Toxicity of Lead and Mercury to Frogs *Rana cyamophlyotis* and *Rana tigerina*," *Environment and Ecology*, Vol 6, 1988, pp. 506–507.

(23) Taylor, D. H, Steele, C. W., and Strickler-Shaw, S., "Responses of Green Frog (*Rana clamitans*) Tadpoles to Lead-Polluted Water, " *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 87–93.

(24) Horne, M. T. and Dunson, W. A., "Effects of Low pH, Metals and Water Hardness on Larval Amphibians," *Arch. Enviro. Contam. Toxicol*, Vol 29, 1995, pp. 500–505.

(25) Fioramonti, E., Semlitsch, R. D., Reyer, Heinz-Ulrich, and Fent, K., "Effects of Triphenyltin and pH on the Growth and Development of *Rana lessonae* and *Rana esculenta* Tadpoles," *Environmental Toxicology and Chemistry*, Vol 16, 1997, pp. 1940–1947.

(26) Hall Jr., L. W., Scott, M. C., and Killen, W. D., "Ecological Risk Assessment of Copper and Cadmium in Surface Waters of Chesapeake Bay Watershed," *Environmental Toxicology and Chemistry*, Vol 17, 1998, pp. 1172–1189.

(27) Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C., Werry, K., and Veldhoen, N., "Toxicity of Glyphosate-based Pesticides to Four North American Frog Species," *Environmental Toxicology and Chemistry*, Vol 23, 2004, pp. 1928–1938.

(28) Jung, R. E. and Walker, M. K., "Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) on Development of Anuran Amphibians," *Environmental Toxicology and Chemistry*, Vol 16, 1997, pp. 230–240.

(29) Allran, J. W. and Karasov, W. H., "Effects of Atrazine and Nitrate on Northern Leopard Frog (*Rana pipiens*) Larvae Exposed in the Laboratory from Posthatch through Metamorphosis," *Environmental Toxicology and Chemistry*, Vol 19, 2000, pp. 2850–2855.

(30) Wojtaszek, B. F., Buscarini, T. M., Chartrand, D. T., Stephenson, G. R., and Thompson, D. G., "Effect of Release® Herbicide on Mortality, Avoidance Response, and Growth of Amphibian Larvae in Two Forest Wetlands," *Environmental Toxicology and Chemistry*, Vol 24, 2005, pp. 2533–2544.

(31) Fort, D. J., Propst, T. L., Stover, E. L., Helgen, J. C., Levey, B., Gallagher, K., and Burkhart, J. G., "Effects of Pond Water, Sediment, and Sediment Extracts from Minnesota and Vermont, USA, on Early Development and Metamorphosis of *Xenopus*," *Environmental Toxicology and Chemistry*, Vol 18, 1999, pp. 2305–2315.

(32) Savage, W. K., Quimby, F. W., and DeCaprio, A. P., "Lethal and Sublethal Effects of Polychlorinated Biphenyls on *Rana sylvatica* Tadpoles," *Environmental Toxicology and Chemistry*, Vol 21, 2002, pp. 168–174.

(33) Widder, P. D. and Bidwell, J. R., "Cholinesterase Activity and Behavior in Chlorpyrifos-exposed *Rana sphenocephala* Tadpoles," *Environmental Toxicology and Chemistry*, Vol 25, 2006, pp. 2446–2454.

(34) U.S. Environmental Protection Agency, "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms-Fourth Edition," EPA-821-R-02-013, 2002.

(35) U.S. Environmental Protection Agency, "National Recommended Water Quality Criteria," United States Environmental Protection Agency, 2006, p. 24.

(36) Chapman, P. M., "Current Approaches to Developing Sediment Quality Criteria," *Environmental Toxicology and Chemistry*, Vol 8, 1989, pp. 589–599.

(37) Di Toro, D. M., Zarba, C. S., Hansen, D. J., Berry, W. J., Swartz, R. C., Cowan, C. E., Pavlou, S. P., Allen, H. E., Thomas, N. A., and Paquin, P. R., "Technical Basis for Establishing Sediment Quality Criteria for Nonionic Organic Chemicals Using Equilibrium Partitioning," *Environmental Toxicology and Chemistry*, Vol 10, 1991, pp. 1541–1583.

(38) Maloney, J., "Influence of Organic Enrichment on the Partitioning and Bioavailability of Cadmium in a Microcosm Study," *Marine Ecology Progress Series*, Vol 144, 1996, pp. 147–161.

(39) Granberg, M. E. and Forbes, T. L., "Role of Sediment Organic Matter Quality and Feeding History in Dietary Absorption and Accumulation of Pyrene in the Mud Snail (*Hydrobia ulvae*)," *Environmental Toxicology and Chemistry*, Vol 25, 2006, pp. 995–1006.

(40) Ankley, G. T., Benoit, D. A., Balogh, J. C., Reynoldsen, T. B., Day, K. E., and Hoke, R. A., "Evaluation of Potential Confounding Factors in Sediment Toxicity Tests with Three Freshwater Benthic Invertebrates," *Environmental Toxicology and Chemistry*, Vol 13, 1994, pp. 627–635.

(41) Sibley, P. K., Ankley, G. T., Cotter, A. M. and Leonardi, E. N., "Predicting Chronic Toxicity of Sediments Spiked with Zinc: An Evaluation of the Acid Volatile Sulfide (AVS) Model Using a Lifecycle Test with the Midge (*Chironomus tentans*)," *Environmental Toxicology and Chemistry*, Vol 15, 1996, pp. 2102–2112.

(42) Ditsworth, G. R. and Schults, D. W., "Preparation of Benthic Substrates for Sediment Toxicity Testing," *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 1523–1529.

(43) Hourdry, J., L'Hermite, A., and Ferrand, R., "Changes in the Digestive Tract and Feeding Behavior of Anuran Amphibians During Metamorphosis," *Physiological Zoology*, Vol 69, 1996, pp. 219–251.

(44) Gosner, K. L., "A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification," *Herpetologica*, Vol 16, 1960, pp. 183–190.

(45) Stemmer, B. L., Burton Jr., G. A., and Sasson-Bricksen, G., "Effect of Sediment Spatial Variance and Collection Method on Cladoceran Toxicity and Indigenous Microbial Activity Determinations," *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 1035–1044.

(46) Sarda, N. and Burton Jr., G. A., "Ammonia Variation in Sediments: Spatial, Temporal and Method-Related Effects," *Environmental Toxicology and Chemistry*, Vol 14, 1995, pp. 1499–1506.

(47) Landrum, P. F., Eadie, B. J., and Faust, W. R., "Variation in the Bioavailability of Polycyclic Aromatic Hydrocarbons to the Amphipod Diporeia with Sediment Aging," *Environmental Toxicology and Chemistry*, Vol 11, 1992, pp. 1197–1208.

(48) Birge, W. J., Westerman, A. G., and Spromberg, J. A., "Comparative Toxicology and Risk Assessment of Amphibians," *Ecotoxicology of Amphibians and Reptiles*, Sparling, Linder and Bishop, eds., Society of Environmental Toxicology and Chemistry, Pensacola, FL, 2000, pp. 727–791.

(49) Karasov, W. H., Jung, R. E., Langenberg, S. V., and Bergeson, T. L. E., "Field Exposure of Frog Embryos and Tadpoles Along a Pollution Gradient in the Fox River and Green Bay Ecosystem in Wisconsin, USA," *Environmental Toxicology and Chemistry*, Vol 24, 2005, pp. 942–953.

(50) Denton, D. L. and Norberg-King, T J., "Whole Effluent Toxicity Statistics: A Regulatory Perspective," *Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts*, Grothe, Dickson, and Reed-Judkins, eds., Society of Environmental Toxicology and Chemistry, Pensacola, FL, 1996, pp. 83–102.

(51) Shumway, W., "Stages in the Normal Development of Rana pipiens," *The Anatomical Record*, Vol 78, 1940, pp. 139–148.

(52) WEST, Inc. and D. D. Gulley, "Toxstat Version 3.5," Western Ecosystems Technology, Inc. Cheyenne, WY, 1996.

(53) Analytical Software, "Statistix 7 for Windows," Analytical Software, Tallahassee, FL, 2000.
(54) U.S. Environmental Protection Agency, "U.S. EPA Toxicity Data Analysis Software. Version 1.5," Environmental Monitoring Systems Laboratory, Cincinnati, OH, 1994.
(55) U.S. Environmental Protection Agency, "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms–Fifth Edition," EPA-821-R-02-012, 2002.
(56) Norberg-King, T., "A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach (Version 2.0)," National Effluent Toxicity Assessment Center Technical Report 03-93, U.S. Environmental Protection Agency, Duluth, MN, 1993.