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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~~Lithobates pipiens*). Other anuran species (for example, the green frog (*~~Rana~~Lithobates clamitans*), the wood frog (*~~Rana~~Lithobates 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 (**8-10~~, 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~~20**). Interestingly, some studies with anurans have shown significantly reduced growth (for example, whole body mass, snout-vent length) can be detected earlier in a longer-term test (for example, at 14-20 d), but cannot be statistically distinguished in older organisms later in the test **(11, 14).** In the development of these procedures, an attempt was made to balance

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

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~~21**). 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 and state/federal regulators involved in the review and approval of risk assessment work plans and reports (**~~13~~22**). To develop this method, a number of tests with spiked sediment tests were conducted (**~~13~~22, ~~14~~23**). Since development of the methodology it has been used operationally to evaluate field-collected sediments from several state and federal environmental sites (**~~15~~24, ~~16~~25**). For most of these studies the preferred test organisms, ~~*Rana*~~*Lithobates 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*~~*Lithobates pipiens*(**~~17~~26**), The test method was also used to evaluate sediment toxicity at a cadmium-contaminated USEPA Region 4-led site in Tennessee (**~~18~~27**). 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 (**~~19-28-29~~40**). Fewer studies have focused on exposure of anuran larvae to sediments, and the methods employed vary widely, from *in situ* enclosures (**~~30~~15, 41**) to laboratory tests using variable exposure conditions and organism ages (**4, 8, 14, 39, ~~31-42-33~~44**). 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~~39, 42,** Guide E1439-98 Appendix X2) to 12 d (**~~33~~44**) and through metamorphosis (**8, ~~32~~14, 43**). Sediment toxicity tests with anurans native to North America were started with larval tadpoles between Gosner stages 23 and 25 (**8, ~~32~~43, ~~33~~44**). Test temperatures were between ~~21~~21 °C and ~~23°C~~23 °C and feeding began after tadpoles reached Gosner stage 25. Food sources were ~~Tetramin™~~ TetraMin[3] (**8**), boiled romaine lettuce (**~~32~~43**), boiled romaine lettuce and flaked fish food (**14**), or boiled romaine lettuce and dissipated rabbit food pellets (**~~33~~44**). Tests were conducted in static renewal mode with water replacements conducted at varying rates (daily (**~~31~~42, ~~33~~44**), weekly (**8**), every 3 to 5 d (**~~32~~43**)). 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~~14, 43**), large bins (**8**), or swimming pools (**~~33~~44**). Endpoints evaluated at test termination included survival (**4, 8, 14, ~~31-42-33~~44**), growth (**8, 14, ~~31-42-33~~44**), bioaccumulation of metals (**8**), developmental rates (**8, ~~32~~14, 43**), deformities (**~~31~~14, ~~32~~42, 43**), swimming speed (**~~33~~44**) and foraging activity levels (**~~32~~43**).

1.6.2 To assess the effect of direct contact with the sediments containing PCBs, Savage et al. (**~~32~~43**) 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. Fuentes et al. (39) evaluated the acute toxicity of two Roundup[4] (a widely used herbicide with the active ingredient glyphosate) formulations to six anuran species, including *Lithobates pipiens,* ~~The~~ under both water-only and water-+sediment conditions. The study found that toxicity of the glyphosate-based herbicides was reduced in the presence of sediment, likely due to sorption to sediment particles and associated organic matter. 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

[3] TetraMin is a trademark of TETRA GMBH.
[4] Roundup is a registered trademark of Monsanto Company.

((~~31~~42~~,~~), 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 Amphibian species may be key receptors of potential chemicals of concern at contaminated sites. Although historically not often included in risk assessments, the importance of amphibians as both sensitive and keystone species is increasingly recognized, particularly considering the decline in amphibian worldwide populations, which may be driven by multiple localized stress agents rather than a single, dominating cause (**45**). The lack of amphibian representation as surrogate species is likely due to multiple factors including scant knowledge of local amphibian populations and life histories, the paucity of applicable toxicity data, and inconsistency in standardized assessment protocols. A review of ecological risk assessment methods for amphibians and gaps in existing amphibian toxicity data and methods is provided by Johnson et al. (**46**). The importance of amphibians in the ecological risk assessment process is recognized by Environment and Climate Change Canada in the Ecological Risk Assessment Guidance under the Federal Contaminated Sites Action Plan (**47**). 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~~ safety, health, and ~~health~~environmental practices and determine the applicability of regulatory limitations prior to use.*

1.11 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[5]

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 Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
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
E1733 Guide for Use of Lighting in Laboratory Testing
E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[6]
SI10-02 IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern

[5] 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.

[6] The last approved version of this historical standard is referenced on www.astm.org.

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~~Guides E943, E1023, E1192, E1367, and E1525. For an explanation of units and symbols, refer to SI10-02 IEEE/ASTM SI 10.

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~~48**)).

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~~49**)) are not exceeded by water-borne concentrations, organisms that live in or near the sediment may still be adversely affected (**~~36~~50**). 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

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.

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~~51), although research also suggests that amending sediment with organic matter actually increases the bioaccumulation of contaminant particles (~~38~~52, ~~39~~53). Other factors that can potentially influence sediment bioaccumulation and toxicity include pH mineralogical composition, acid-volatile sulfide (AVS) ~~and grain size~~ grain size, and temperature (~~40~~54-56, ~~41~~). Laboratory toxicity tests provide a direct and effective way to evaluate the ~~effects~~impacts 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~~39, ~~41-43~~41, 43, 55, 57, 58). 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*~~*Lithobates pipiens*,*Bufo americanus*, ~~*Rana*~~*Lithobates clamitans*,~~*Rana*~~*Lithobates palustris* (pickerel frog), ~~*Rana*~~*Lithobates 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~~59**), 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.

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

TABLE 2 General Equipment Required for Conducting a 10-d Sediment Toxicity Test with *~~Rana~~Lithobates 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 ± 1°C~~
Temperature-controlled water bath or environmental chamber capable of controlling to 23 °C ± 1 °C
Flow-through water delivery system
Buffered 3-aminobenzoic acid ethyl ester, methanesulfonate salt (MS-222 anesthetic) solution.
~~Food source (TetraMin™)~~
Food source (dried fish food flakes)
Appropriate data forms
Metric ruler
Forceps
Statistical software

the development of the sampling/processing ~~procedure.~~ procedure (see Practice E1847). 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~~60), 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~~61**). 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~~60**) and aging (**~~47~~62**) 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~~22**) were previously employed for incorporation of both inorganic contaminants and organic chemicals into sediment (**~~42~~57**). 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 (**39, ~~37~~-51-~~41~~56**). 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.*~~*L. pipiens*. Sediment toxicity testing conducted with both ~~*R.*~~*L. pipiens* and the American toad, *B. americanus*, during the development of this standard indicated that ~~*R.*~~*L. pipiens* was generally more sensitive to spiked sediments containing metals (cadmium, copper, lead, or zinc) than was *B. americanus*(**~~13~~22**). A review of amphibian data presented in U.S. EPA ambient water quality criteria documents for cadmium, copper, and zinc (**~~13~~22**) and relative sensitivity data evaluating amphibian aquatic LC50s (**~~48~~63**) indicate that ~~*R.*~~*L. pipiens* is considered to be sensitive to metals, relative to other frog, toad, and salamander species. Other ranid species (~~*R. catesbeiana,*~~*L. catesbeianus,*~~*R.*~~*L. palustris*) were also sensitive to the metals reviewed (**~~13~~22, ~~48~~63**). The potential for field-collection of ~~*R.*~~*L. 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.*~~*L. 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.*~~*L. 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 be 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~~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 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 ~~1~~5 % / hour to 25 % / hour for 4 hours 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.

TABLE 3 Developmental Stages of Anuran Embryos (from Gosner (44~~59~~) and Shumway (49~~64~~))

Stage	Approximate Age at ~~18°C~~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 ~ ⅕ 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

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.~~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.,~~ (for example, 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,~~,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.

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

Parameter	Conditions
1. Test type:	Whole-sediment toxicity test with renewal of overlying water
~~2. Temperature:~~	~~23 ± 1°C~~
2. Temperature:	23 °C ± 1°C
~~3. Light quality:~~	~~Wide-spectrum fluorescent lights~~
3. Light quality:	Wide-spectrum fluorescent or LED lights (see Guide E1733)
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~~
6. Test chamber:	400 mL 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~~
13. Feeding:	4 mg of ground dried fish food flakes 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.