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Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates[1]

This standard is issued under the fixed designation E 1706; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

ϵ[1] NOTE—Section 1.8 was editorially revised in February 2003.

1. Scope*

1.1 This test method covers procedures for testing freshwater organisms in the laboratory to evaluate the toxicity of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory.

1.1.1 Test methods are described for two toxicity test organisms, the amphipod *Hyalella azteca* (*H. azteca*) (see 13.1.2) and the midge *Chironomus tentans* (*C. tentans*) (see 14.1.2). The toxicity tests are conducted for 10 days in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and test organisms are fed during the toxicity tests. Endpoints for the 10-day toxicity tests are survival and growth. These test methods describe procedures for testing freshwater sediments; however, estuarine sediments (up to 15 ppt salinity) can also be tested with *H. azteca.* In addition to the 10-day toxicity test method outlined in 13.1.2 and 14.1.2, general procedures are also described for conducting 10-day sediment toxicity tests with *H. azteca* (see 13.1.2) and *C. tentans* (see 14.1.2).

1.1.2 Guidance for conducting sediment toxicity tests is outlined in Annex A1 for *Chironomus riparius,* in Annex A2 for *Daphnia magna* and *Ceriodaphnia dubia,* in Annex A3 for *Hexagenia* spp., in Annex A4 for *Tubifex tubifex,* and in Annex A5 for the *Diporeia* spp. Guidance is also provided in Annex A6 for conducting long-term sediment toxicity tests with *H. azteca* by measuring effects on survival, growth, and reproduction. Guidance is also provided in Annex A7 for conducting long-term sediment toxicity tests with *C. tentans* by measuring effects on survival, growth, emergence, and reproduction. 1.6 outlines the data that will be needed before test methods are developed from the guidance outlined in Annex A1 to Annex A7 for these test organisms. General procedures described in Sections 17 for sediment testing with *H. azteca* and *C. tentans* are also applicable for sediment testing with the test organisms described in Annex A1 to Annex A7.

1.2 Procedures outlined in this test method are based primarily on procedures described in the United States Environmental Protection Agency (USEPA) (**1-8**)[2] and Guides E 1367, E 1391, E 1525 and E 1688.

1.3 Additional research and methods development are now in progress to: *(1)* evaluate additional test organisms, *(2)* further evaluate the use of formulated sediment, *(3)* refine sediment dilution procedures, *(4)* refine sediment toxicity identification evaluation (TIE) procedures (**9**), *(5)* refine sediment spiking procedures, *(6)* develop *in situ* toxicity tests to assess sediment toxicity and bioaccumulation under field conditions, *(7)* evaluate relative sensitivities of endpoints measured in tests, *(8)* develop methods for new species, *(9)* evaluate relationships between toxicity and bioaccumulation, and *(10)* produce additional data on confirmation of responses in laboratory tests with natural populations of benthic organisms. Some issues that may be considered in interpretation of test results are the subject of continuing research including the influence of feeding on bioavailability, nutritional requirements of the test organisms, and additional performance criteria for organism health. See Section 6 for additional detail. This information will be described in future editions of this standard.

1.4 The USEPA (**1**) and Guide E 1688 also describes 28-day bioaccumulation methods for the oligochaete *Lumbriculus variegatus*.

1.5 Results of tests, even those with the same species, using procedures different from those described in the test method may not be comparable and using these different procedures may alter bioavailability. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms. If tests are conducted with procedures different from those described in this test method, additional tests are required to determine comparability of results. General procedures described in this test method might be useful for conducting tests with other aquatic organisms; however, modifications may be necessary.

[1] This test method is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and are the direct responsibility of Subcommittee E47.03 on Sediment Assessment and Toxicology.

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

***A Summary of Changes section appears at the end of this standard.**

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1.6 *Selection of Toxicity Testing Organisms*:

1.6.1 The choice of a test organism has a major influence on the relevance, success, and interpretation of a test. Furthermore, no one organism is best suited for all sediments. The following criteria were considered when selecting test organisms to be described in this standard (Table 1 and Guide E 1525). A test organism should: *(1)* have a toxicological data base demonstrating relative sensitivity and discrimination to a range of chemicals of concern in sediment, *(2)* have a database for interlaboratory comparisons of procedures (for example, round-robin studies), *(3)* be in contact with sediment [e.g., water column vs benthic organisms], *(4)* be readily available through culture or from field collection, *(5)* be easily maintained in the laboratory, *(6)* be easily identified, *(7)* be ecologically or economically important, *(8)* have a broad geographical distribution, be indigenous (either present or historical) to the site being evaluated, or have a niche similar to organisms of concern, (for example, similar feeding guild or behavior to the indigenous organisms), *(9)* be tolerant of a broad range of sediment physico-chemical characteristics (for example, grain size), and *(10)* be compatible with selected exposure methods and endpoints. The method should also be *(11)* peer reviewed and *(12)* confirmed with responses with natural populations of benthic organisms (see 1.6.8).

1.6.2 Of the criteria outlined in Table 1, a data base demonstrating relative sensitivity to contaminants, contact with sediment, ease of culture in the laboratory, interlaboratory comparisons, tolerance of varying sediment physico-chemical characteristics, and confirmation with responses of natural benthos populations were the primary criteria used for selecting *H. azteca* and *C. tentans* to be described as test methods in the current version of this standard (see Sections 13 and 14). Procedures for conducting sediment tests with organisms in accordance with Annex A1 to Annex A7 do not currently meet all the required selection criteria listed in Table 1. A similar data base must be developed before these or other test organisms can be included as standard test methods instead of as guidance in future versions of these this method.

1.6.3 An important consideration in the selection of specific species for test method development is the existence of information concerning relative sensitivity of the organisms both to single chemicals and complex mixtures. A number of studies have evaluated the sensitivity of *H. azteca, C. tentans*, and *L. variegatus,* relative to one another, as well as other commonly tested freshwater species. For example, Ankley et al **(10)** found *H. azteca* to be as, or slightly more, sensitive than *Ceriodaphnia dubia* to a variety of sediment elutriate and pore-water samples. In that study, *L. variegatus* were less sensitive to the samples than either the amphipod or the cladoceran. West et al **(11)** found the rank sensitivity of the three species to the lethal effects of copper in sediments from the Keweenaw Waterway, MI was (from greatest to least): *H. azteca* > *C. tentans* > *L. variegatus*. In short-term (48 to 96 h) exposures, *L. variegatus* generally was less sensitive than *H. azteca, C. dubia,* or *Pimephales promelas* to cadmium, nickel, zinc, copper, and lead **(12)**. Of the latter three species, no one species was consistently the most sensitive to the five metals.

1.6.3.1 In a study of contaminated Great Lakes sediment, *H. azteca, C. tentans,* and *C. riparius* were among the most sensitive and discriminatory of 24 organisms tested **(13-16)**. Kemble et al **(17)** found the rank sensitivity of four species to metal-contaminated sediments from the Clark Fork River, MT to be (from greatest to least): *H. azteca* > *C. riparius* > *Oncorhynchus mykiss* (rainbow trout) > *Daphnia magna*. Relative sensitivity of the three endpoints evaluated in the *H. azteca* test with Clark Fork River sediments was (from greatest to least): length > sexual maturation > survival.

1.6.3.2 In 10-day water-only and whole-sediment tests, *Hyalella azteca* and *C. tentans* were more sensitive than *D. magna* to fluoranthene-spiked sediment **(18)**.

1.6.3.3 Ten-day, water-only tests also have been conducted with a number of chemicals using *H. azteca, C. tentans,* and *L. variegatus* (**(18)** and Table 2). These tests all were flow-through exposures using a soft natural water (Lake Superior) with measured chemical concentrations that, other than the absence of sediment, were conducted under conditions (for example, temperature, photoperiod, feeding) similar to those being described for the standard 10-day sediment test in 13.1.2. In general, *H. azteca* was more sensitive to copper, zinc, cadmium, nickel, and lead than either *C. tentans* or *L. variegatus. Chironomus tentans* and *H. azteca* exhibited a similar sensitivity to several of the pesticides tested. *Lumbriculus variegatus* was not tested with several of the pesticides; however, in other studies with whole sediments contaminated by dichlorodiphenyltrichloroethane (DDT) and associated metabolites, and in short-term (96-h) experiments with organophosphate insecticides (diazinon, chlorpyrifos), *L. variegatus* has proved to be far less sensitive than either *H. azteca* or *C. tentans*. These results highlight two important points germane to these test methods. First, neither of the two test species selected for estimating sediment toxicity (*H. azteca, C. tentans*) was consistently most sensitive to all chemicals, indicating the importance of using multiple test organisms when performing sediment assessments. Second, *L. variegatus* appears to be relatively insensitive to most of the test chemicals, which perhaps is a positive attribute for an organism used for bioaccumulation testing **(9)**.

1.6.3.4 Using the data from Table 2, sensitivity of *H. azteca, C. tentans*, and *L. variegatus* can be evaluated relative to other freshwater species. For this analysis, acute and chronic toxicity data from water quality criteria (WQC) documents for copper, zinc, cadmium, nickel, lead, DDT, dieldrin, and chlorpyrifos, and toxicity information from the AQUIRE data base **(19)** for 1,1,dichloro-2,2-bis(p-chlorophenyl)ethane (DDD) and dichlorodiphenyldichloroethylene (DDE), were compared to assay results for the three species **(18)**. The sensitivity of *H. azteca* to metals and pesticides, and *C. tentans* to pesticides was comparable to chronic toxicity data generated for other test species. This was not completely unexpected given that the 10-day exposures used for these two species are likely more similar to chronic partial life-cycle tests than the 48 to 96-h exposures traditionally defined as acute in the WQC documents. Interestingly, in some instances (for example, dieldrin and chlorpyrifos), LC50 data generated for *H. azteca* or *C. tentans* were comparable to or lower than any reported for other freshwater

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species in the WQC documents. This observation likely is a function not only of the test species, but of the test conditions; many of the tests on which early WQC were based were static, rather than flow-through, and report unmeasured contaminant concentrations.

1.6.3.5 Measurable concentrations of ammonia are common in the pore water of many sediments and have been found to be a common cause of toxicity in pore water (**20 21, 22**). Acute toxicity of ammonia to *H. azteca, C. tentans*, and *L. variegatus* has been evaluated in several studies. As has been found for many other aquatic organisms, the toxicity of ammonia to *C. tentans* and *L. variegatus* has been shown to be dependent on pH. Four-day LC50 values for *L. variegatus* in water-column (no sediment) exposures ranged from 390 to 6.6 mg/L total ammonia as pH was increased from 6.3 to 8.6 Schubauer-Berigan et al.(**23**). For *C. tentans*, 4-day LC50 values ranged from 370 to 82 mg/L total ammonia over a similar pH range (Schubauer-Berigan et al.) (**23**). Ankley et al. (**24**) reported that the toxicity of ammonia to *H. azteca* (also in water-only exposures) showed differing degrees of pH-dependence in different test waters. In soft reconstituted water, toxicity was not pH dependent, with 4-day LC50 values of about 20 mg/L at pH ranging from 6.5 to 8.5. In contrast, ammonia toxicity in hard reconstituted water exhibited substantial pH dependence with LC50 values decreasing from >200 to 35 mg/L total ammonia over the same pH range. Borgmann and Borgmann (**25**) later showed that the variation in ammonia toxicity across these waters could be attributed to differences in sodium and potassium content, which appear to influence the toxicity of ammonia to *H. azteca.*

1.6.3.5.1 Although these studies provide benchmark concentrations that may be of concern in sediment pore waters, additional studies by Whiteman et al. (**26**) indicated that the relationship between water-only LC50 values and those measured in sediment exposures differs among organisms. In sediment exposures, the 10-day LC50 for *L. variegatus* and *C. tentans* occurred when sediment pore water reached about 150 % of the LC50 determined from water-only exposures. However, experiments with *H. azteca* showed that the 10-day LC50 was not reached until pore water concentrations were nearly 10× the water-only LC50, at which time the ammonia concentration in the overlying water was equal to the water-only LC50. The authors attribute this discrepancy to avoidance of sediment by *H. azteca*. Thus, it appears that water-only LC50 values may provide suitable screening values for potential ammonia toxicity, higher concentrations may be necessary to actually induce ammonia toxicity in sediment exposures, particularly for *H. azteca*. Further, these data underscore the importance of measuring the pH of pore water when ammonia toxicity may be of concern. Ankley Schubauer-Bergian (**27**) and Besser et al. (**28**) describe procedures for conducting toxicity identification evaluations (TIEs) for pore-water or whole-sediment samples to determine if ammonia is contributing to the toxicity of sediment samples.

1.6.4 Relative species sensitivity frequently varies among chemicals; consequently, a battery of tests including organisms representing different trophic levels may be needed to assess sediment quality (**13, 16, 29-32**). For example, Reish (**33**) reported the relative toxicity of six metals (arsenic, cadmium, chromium, copper, mercury, and zinc) to crustaceans, polychaetes, pelecypods, and fishes and concluded that no one species or group of test organisms was the most sensitive to all of the metals.

1.6.4.1 Sensitivity of a species to chemicals is also dependent on the duration of the exposure and the endpoints evaluated. Annex A6 and Annex A7 describe results of studies which demonstrate the utility of measuring sublethal endpoints in sediment toxicity tests with the amphipod *H. azteca* and the midge *C. tentans*.

1.6.5 The sensitivity of an organism to chemicals should be balanced with the concept of discrimination (**13**). The response of a test organism should provide discrimination between different levels of contamination. However, insensitive organisms may be preferred for determining bioaccumulation. The use of indigenous organisms that are ecologically important and easily collected is often very straightforward; however, indigenous organisms at a site may be insensitive to the chemicals of concern. Indigenous organisms might be more important for evaluation of bioaccumulation (**9**). See Guides E 1525, E 1688, and E 1850 for additional detail on selection of test organisms.

1.6.6 Sensitivity of an organism is related to route of exposure and biochemical sensitivity to chemicals. Sediment-dwelling organisms can receive a dose from three primary sources: interstitial water, sediment particles, and overlying water. Food type, feeding rate, assimilation efficiency, and clearance rate will control the dose of chemicals from sediment (Guide E 1688). Benthic invertebrates often selectively consume different particle sizes (**34**) or particles with higher organic carbon concentrations which may have higher chemical concentrations. Detrital feeders may receive most of their body burden directly from sediment ingestion. In amphipods (**35**) and clams (**36**) uptake through the gut can exceed uptake across the gills for certain hydrophobic compounds. Organisms in direct contact with sediment may also accumulate chemicals by direct adsorption to the body wall or by absorption through the integument (**37**).

1.6.7 Despite the potential complexities in estimating the dose that an animal receives from sediment, the toxicity and bioaccumulation of many chemicals in sediment such as chlordecone, fluoranthene, organochlorines, and metals have been correlated with either the concentration of these chemicals in interstitial water or in the case of nonionic organic chemicals, concentrations of an organic-carbon basis (**38, 39**). The relative importance of whole sediment and interstitial water routes of exposure depends on the test organism and the specific contaminant (**34, 37**). Because benthic communities contain a diversity of organisms, many combinations of exposure routes may be important. Therefore, behavior and feeding habits of a test organism can influence its ability to accumulate contaminants from sediment and should be considered when selecting test organisms for sediment testing.

1.6.8 The response of *H. azteca* and *C. tentans* in laboratory toxicity studies has been compared to the response of natural populations of benthic organisms to potentially contaminated sediments.

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1.6.8.1 Chironomids were not found in sediment samples that decreased the growth of *C. tentans* by 30 % or more in 10-day laboratory toxicity tests (**40**). Wentsel et al (**41-43**) reported a correlation between effects on *C. tentans* in laboratory tests and the abundance of *C. tentans* in metal-contaminated sediments.

1.6.8.2 Canfield et al. (**44,45,46**) evaluated the composition of benthic invertebrate communities in sediments for the following areas: (1) three Great Lakes Areas of Concern (AOC; Buffalo River, NY: Indiana Harbor, IN: Saginaw River, MI), (2) the upper Mississippi River, and (3) the Clark Fork River located in Montana. Results of these benthic community assessments were compared to sediment chemistry and toxicity (28-day sediment exposures with *H. azteca* which monitored effects on survival, growth, and sexual maturation). Good concordance was evident between measures of laboratory toxicity, sediment contamination, and benthic invertebrate community composition in extremely contaminated samples. However, in moderately contaminated samples, less concordance was observed between the composition of the benthic community and either laboratory toxicity test results or sediment contaminant concentration. Laboratory sediment toxicity tests better identified chemical contamination in sediments compared to many of the commonly used measures of benthic invertebrate community composition. Benthic measures may reflect other factors such as habitat alteration in addition to responding to contaminants. Canfield et al. (**44, 45, 46**) identified the need to better evaluate non-contaminant factors (i.e., TOC, grain size, water depth, habitat alteration) in order to better interpret the response of benthic invertebrates to sediment contamination.

1.6.8.3 Results from laboratory sediment toxicity tests were compared to colonization of artificial substrates exposed *in situ* to Great Lakes sediment (**13**) Burton et al. (**16**) Survival or growth of *H. azteca* and *C. tentans* in 10–28-day laboratory exposures were negatively correlated to percent chironomids and percent tolerant taxa colonizing artificial substrates in the field. Schlekat et al (**47**) reported general good agreement between sediment toxicity tests with *H. azteca* and benthic community responses in the Anacostia River in Washington, DC.

1.6.8.4 Sediment toxicity with amphipods in 10-day toxicity tests, field contamination, and field abundance of benthic amphipods were examined along a sediment contamination gradient of DDT (**47**). Survival of *Eohaustorius estuarius, Rhepoxynius abronius*, and *H. azteca* in laboratory toxicity tests was positively correlated to abundance of amphipods in the field and negatively correlated to DDT concentrations. The threshold for 10-day sediment toxicity in laboratory studies was about 300 µg DDT (+metabolites)/g organic carbon. The threshold for abundance of amphipods in the field was about 100 µg DDT (+metabolites)/g organic carbon. Therefore, correlations between toxicity, contamination, and field populations indicate that short-term sediment toxicity tests can provide reliable evidence of biologically adverse sediment contamination in the field, but may be underprotective of sublethal effects.

1.7 *Limitations*— While some safety considerations are included in this standard, it is beyond the scope of this standard to encompass all safety requirements necessary to conduct sediment tests.

1.8 *This standard is arranged as follows*:

1 Scope
2 Referenced Documents
3 Terminology
4 Summary of Standard
5 Significance and Use
6 Interferences
7 Reagents and Materials
8 Hazards
9 Facilities, Equipment, and Supplies
10 Sample Collection, Storage, Manipulation, and Characterization
11 Quality Assurance and Quality Control
12 Collection, Culturing, and Maintaining Test Organisms
13 Procedure 1: Conducting a 10-day Sediment Toxicity Test with *Hyalella azteca*
14 Procedure 2: Conducting a 10-day Sediment Toxicity Test with *Chironomus tentans*
15 Calculation
16 Report
17 Precision and Bias
18 Keywords
Annexes
A1. Guidance for Conducting Sediment Toxicity Tests with *Chironomus riparius*
A2. Guidance for Conducting Sediment Toxicity Tests with *Daphniaus magna* and *Ceriodaphnia dubia*
A3. Guidance for Conducting Sediment Toxicity Tests with *Hexagenia spp.*
A4. Guidance for Conducting Sediment Toxicity Tests with *Tubifex tubifex*
A5. Guidance for Conducting Sediment Toxicity Tests with *Diporeia spp.*
A6. Guidance for Conducting a Hyalella Azteca 42-day Test for Measuring Effects of Sediment-Associated Contaminants on Survival, Growth, and Reproduction
A7. Guidance for Conducting a Life-Cycle Test for Measuring Effects of Sediment-Associated Contaminants on *Chironomus tentans*.
A8. Food Preparation
A9. Feeding Rate for the 10-day Sediment Toxicity Test Method with *Chironomus tentans*
References

1.9 *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.* Specific hazard statements are given in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[3]

D 4387 Guide for Selecting Grab Sampling Devices for Collecting Benthic Macroinvertebrates[4]

D 4447 Guide for the Disposal of Laboratory Chemicals and Samples[5]

E 29 Practice for Using Significant Digits in Test Data to Determine Conformance with Specifications[6]

E 105 Practice for Probability Sampling of Materials[6]

E 122 Practice for Choice of Sampling Size to Estimate a Measure of Quality for a Lot or Process[6]

[3] *Annual Book of ASTM Standards,* Vol 11.01.
[4] *Annual Book of ASTM Standards,* Vol 11.05.
[5] *Annual Book of ASTM Standards,* Vol 11.04.
[6] *Annual Book of ASTM Standards,* Vol 14.02.

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TABLE 1 Rating of Selection Criteria for Freshwater Sediment Toxicity Testing Organisms. A "+" or "–" Rating Indicates a Positive or Negative Attribute ("NA" = Not Applicable)

Criterion	*Hyalella azteca*	*Diporeia spp.*	*Chironomus tentans*	*Chironomus riparius*	*Lumbriculus variegatus*	*Tubifex tubifex*	*Hexagenia spp.*	Molluscs	*Daphnia spp.* and *Ceriodaphnia spp.*
Relative sensitivity toxicity data base	+	–	+	–	+	–	–	–	–
Round-robin studies conducted	+	–	+	–	–	–	–	–	–
Contact with sediment	+	+	+	+	+	+	+	+	–
Laboratory culture	+	–	+	+	+	+	–	–	+
Taxonomic identification	+	+/–	+/–	+/–	+	+	+	+	+
Ecological importance	+	+	+	+	+	+	+	+	+
Geographical distribution	+	+/–	+	+	+	+	+	+	+/–
Sediment physicochemical tolerance	+	+	+/–	+	+	+	–	+	NA
Response confirmed with benthos populations	+	+	+	+	+	+	+	–	+
Peer reviewed	+	+	+	+	+	+	+	–	+/–
Endpoints monitored	S,G,M	S,B,A	S,G,E	S,G,E	B,S	S,R	S,G	B	S,G,R

S = survival, G = Growth, B = Bioaccumulation, A = avoidance
R = Reproduction, M = Maturation, E = Emergence

TABLE 2 Water-Only, 10-Day LC50 (µg/L) Values for *Hyalella azteca, Chironomus tentans*, and *Lumbriculus variegatus* for Chemicals Tested at ERL-Duluth in Soft Water (Hardness 40 mg/L as $CaCO_3$; (18))

Chemical	*H. azteca*	*C. tentans*	*L. variegatus*
Copper	35	54	35
Zinc	73	1125[1]	2984
Cadmium	2.8[2]	NT[3]	158
Nickel	780	NT	12 160
Lead	<16	NT	794
p,p′-DDT	0.07	1.23	NT
p,p′-DDD	0.17	0.18	NT
p,p′-DDE	1.39	3.0	>3.3
Dieldrin	7.6	1.1	NT
Chlorpyrifos	0.086	0.07	NT

[1] 50 % mortality at highest concentration tested.
[2] 70 % mortality at lowest concentration tested.
[3] NT, not tested.

E 141 Practice for Acceptance of Evidence Based on Results of Probability Sampling[6]

E 177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods[6]

E 178 Practice for Dealing with Outlying Observations[6]

E 380 Practice for Use of the International System of Units (SI) (The Modernized Metric System)[6]

E 456 Terminology Relating to Quality and Statistics[6]

E 691 Practice for Conducting an Interlaboratory Study to Determine Precision of a Test Method[6]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[4]

E 943 Terminology Relating to Biological Effects and Environmental Fate[4]

E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*[4]

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes[4]

E 1295 Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*[4]

E 1325 Terminology Relating to Design of Experiments[6]

E 1367 Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods[4]

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing[4]

E 1402 Terminology Relating to Sampling[6]

E 1525 Guide for Designing Biological Tests with Sediments[4]

E 1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates[4]

E 1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines[4]

E 1850 Guide for Section of Resident Species at Test Organisms for Aquatic and Sediment Tests[4]

3. Terminology

3.1 The words "must", "should"," may", "can", and "might" have very specific meanings in this standard. "Must" is used to express an absolute requirement, that is, to state that a test ought to be designed to satisfy the specified conditions, unless the purpose of the test requires a different design. "Must" is used only in connection with the factors that relate directly to the acceptability of a test. "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 will often render the results questionable. Terms such as "is desirable," "is often desirable," and" might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to,"" can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, 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 other terms used in this test method, refer to Guides E 729 and E 1241 and Terminology E 943 and D 1129. For an explanation of units and symbols, refer to Practice E 380.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *clean*—denotes a sediment or water that does not contain concentrations of test materials which cause apparent stress to the test organisms or reduce their survival.

3.3.2 *concentration*—the ratio of weight or volume of test material(s) to the weight or volume of sediment.

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3.3.3 *contaminated sediment*—sediment containing chemical substances at concentrations that pose a known or suspected threat to environmental or human health.

3.3.4 *control sediment*—a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test. Any contaminants in control sediment may originate from the global spread of pollutants and does not reflect any substantial input from local or non-point sources. Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination.

3.3.5 *EC50*—a statistically or graphically estimated concentration that is expected to cause one or more specified effects in 50 % of a group of organisms under specified conditions.

3.3.6 *Formulated sediment*—Mixtures of materials used to mimic the physical components of a natural sediment.

3.3.7 *IC50*—a point estimate of the toxicant concentration that would cause a 50 % reduction in a non-quantal measurement such as fecundity or growth.

3.3.8 *interstitial water or pore water*—water occupying space between sediment or soil particles.

3.3.9 *LC50*—a statistically or graphically estimated concentration that is expected to be lethal to 50 % of a group of organisms under specified conditions.

3.3.10 *lowest-observable-effect concentration (LOEC)*—in a toxicity test, the lowest tested concentration of a material at which organisms were adversely affected compared to control organisms as determined by statistical hypothesis tests—should be accompanied by a description of the statistical tests and alternative hypotheses, levels of significance, and measures of performance, for example, survival, growth, reproduction, or development—and must be above any other concentration not producing statistically significant adverse effects.

3.3.11 *no-observable-effect concentration (NOEC)*— in a toxicity test, the highest tested concentration of a material at which organisms did as well as control organisms as determined by statistical hypothesis tests—should be accompanied by a description of the statistical tests and alternative hypotheses, levels of significance, and measures of performance, for example, survival, growth, reproduction, or development—and must be below any other concentration producing statistically significant adverse effects.

3.3.12 *overlying water*—the water placed over sediment in a test chamber during a test.

3.3.13 *reference sediment*—a whole sediment near an area of concern used to assess sediment conditions exclusive of material(s) of interest. The reference sediment may be used as an indicator of localized sediment conditions exclusive of the specific pollutant input of concern. Such sediment would be collected near the site of concern and would represent the background conditions resulting from any localized pollutant inputs as well as global pollutant input. This is the manner in which reference sediment is used in dredge material evaluations.

3.3.14 *reference-toxicity test*—a test conducted with 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.

3.3.15 *sediment*—particulate material that usually lies below water. Formulated particulate material that is intended to lie below water in a test.

3.3.16 *spiked sediment*—a sediment to which a material has been added for experimental purposes.

3.3.17 *whole sediment*—sediment and associated pore water which have had minimal manipulation. The term bulk sediment has been used synonymously with whole sediment.

4. Summary of Standard

4.1 *Method Description*—Procedures are described for testing freshwater organisms in the laboratory to evaluate the toxicity of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory.

4.1.1 Test methods are described for conducting toxicity tests with two organisms: the amphipod *Hyalella azteca* (see 13.1.2) and the midge *Chironomus tentans* (see 14.1.2). The toxicity tests are conducted for 10 days in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is renewed daily and test organisms are fed during the toxicity tests. Endpoints for the 10-day toxicity tests are survival and growth. Length or weight is reported as the average of the surviving organisms at the end of the test (Sections 13 and 14). Another approach for reporting growth might be as biomass (dry weight of surviving organisms divided by the initial number of organisms). The rationale for evaluating biomass in toxicity testing is that small differences in either growth or survival may not be statistically significantly different from the control; however, a combined estimate of biomass may increase the statistical power of the test. While USEPA (**2**) recommend reporting biomass as a measure of growth in effluent toxicity tests, the approach has not yet been routinely applied in sediment testing. Therefore, biomass is not listed as a primary endpoint in the methods described in Sections 13 and 14 or in Annex A1 to Annex A7. The standard describes procedures for testing freshwater sediments; however, estuarine sediments (up to 15 ppt salinity) can also be tested with *H. azteca*. In addition to the 10-day toxicity test methods outlined in 13.1.2 and 14.1.2, general procedures are also described for conducting sediment toxicity tests with *H. azteca* (see 13.1.2) and *C. tentans* (see 14.1.2).

4.1.2 Guidance for conducting sediment toxicity tests is provided in Annex A1 for *Chironomus riparius,* in Annex A2 for *Daphnia magna* and *Ceriodaphnia dubia,* in Annex A3 for *Hexagenia spp.*, in Annex A4 for *Tubifex tubifex,* and in Annex A5 for the *Diporeia spp.*

4.1.3 Guidance for conducting long-term sediment toxicity tests with *H. azteca* by measuring effects on survival, growth, and reproduction is provided in Annex A6. The long-term sediment exposures with *H. azteca* are started with 7- to 8-day-old amphipods. On Day 28, amphipods are isolated from the sediment and placed in water-only chambers where reproduction is measured on Day 35 and 42. Endpoints measured in the long-term amphipod test include survival (Day 28, 35, and 42), growth (Day 28 and 42), and reproduction (number of young/female produced from Day 28 to 42). Guidance for

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conducting long-term sediment toxicity tests with *C. tentans* by measuring effects on survival, growth, emergence, and reproduction is provided in Annex A7. The long-term sediment exposures with *C. tentans* start with newly hatched larvae (<24-h old) and continue through emergence, reproduction, and hatching of the F_1 generation (about 60-day exposures). Survival and growth are determined at 20 day. Starting on Day 23 to the end of the test, emergence and reproduction of *C. tentans* are monitored daily. The number of eggs/female is determined for each egg case, which is incubated for 6 day to determine hatching success.

4.1.3.1 The long-term toxicity testing methods for *Hyalella azteca* (Annex A6) and *Chironomus tentans* (Annex A7) can be used to measure effects on reproduction as well as long-term survival and growth. Reproduction is a key variable influencing the long-term sustainability of populations (Rees and Crawley, (**48**)) and has been shown to provide valuable and sensitive information in the assessment of sediment toxicity Derr and Zabik, (**49**); Wentsel et al., (**50**); Williams et al., (**51**); Postma et al., (**52**); Sibley et al., (**53**), (**54**); Ingersoll et al., (**55**). Further, as concerns have emerged regarding the environmental significance of chemicals that can act directly or indirectly on reproductive endpoints (e.g., endocrine disrupting compounds), the need for comprehensive reproductive toxicity tests has become increasingly important. Reproductive endpoints measured in sediment toxicity tests with *H. azteca* and *C. tentans* tend to be more variable compared to survival or growth (Section A6.4.6 and A7.5.4.6). Hence, additional replicates would be required to achieve the same statistical power as for survival and growth endpoints (Section 16). The procedures described in Annex A6 and Annex A7 include measurement of a variety of lethal and sublethal endpoints; minor modifications of the basic methods can be used in cases where only a subset of these endpoints is of interest (A6.1.3 and A7.1.2).

4.1.4 Paragraph 1.6 outlines the data that will be needed before test methods are developed from the guidance outlined for these test organisms in Annex A1 to Annex A7. General procedures described in Sections 1 to 14 for sediment testing with *H. azteca* and *C. tentans* are also applicable for sediment testing with the test organisms described in Annex A1 to Annex A7.

4.2 *Experimental Design*—The following section is a general summary of experimental design. See Section 15 for additional detail.

4.2.1 *Control and Reference Sediment*:

4.2.1.1 Sediment tests include a control sediment (sometimes called a negative control). A control sediment is a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test and is not necessarily collected near the site of concern. Any contaminants in control sediment are thought to originate from the global spread of pollutants and do not reflect any substantial inputs from local or non-point sources (**9**). Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination and organism health (**9**). A control sediment provides a measure of test acceptability, evidence of test organism health, and a basis for interpreting data obtained from the test sediments. 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.

4.2.1.1.1 In general, the performance of test organisms in the negative control is used to judge the acceptability of a test, and either the negative control or reference sediment may be used to evaluate performance in the experimental treatments, depending on the purpose of the study. Any study in which organisms in the negative control do not meet performance criteria must be considered questionable because it suggests that adverse factors affected the response of test organisms. Key to avoiding this situation is using only control sediments that have a demonstrated record of performance using the same test procedure. This includes testing of new collections from sediment sources that have previously provided suitable control sediment.

4.2.1.1.2 Because of the uncertainties introduced by poor performance in the negative control, such studies should be repeated to insure accurate results. However, the scope or sampling associated with some studies may make it difficult or impossible to repeat a study. Some researchers have reported 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 resonable to infer that other samples that show good performance are probably not toxic; however, any samples showing poor performance should not be judged to have shown toxicity, since it is unknown whether the adverse factors that caused poor control performance might have also caused poor performance in the test treatments.

4.2.1.2 Natural physico-chemical characteristics such as sediment texture may influence the response of test organisms (**56**). The physico-chemical characteristics of test sediment need to be within the tolerance limits of the test organism. Ideally, the limits of a test organism should be determined in advance; however, controls for factors including grain size and organic carbon can be evaluated if the limits are exceeded in a test sediment. See 12.1 for information on physico-chemical requirements of test organisms. If the physico-chemical characteristics of a test sediment exceed the tolerance range of the test organism, a control sediment encompassing these characteristics can be evaluated. The effects of sediment characteristics on the results of sediment tests can be addressed with regression equations (**56, 57**). The use of formulated sediment can also be used to evaluate physico-chemical characteristics of sediment on test organisms (**58, 59, 60,61**).

4.2.2 The experimental design depends on the purpose of the study. Variables that need to be considered include the number and type of control sediments, the number of treatments and replicates, and water quality characteristics. For instance, the purpose of the study might be to determine a specific endpoint such as an LC50 and may include a control sediment, a positive control, a solvent control, and several concentrations of sediment spiked with a chemical. See Section

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10.3.2). A useful summary of field sampling design is presented by (**62**). See Section 15 for additional guidance on experimental design and statistics.

4.2.2.1 The purpose of the study might be to determine if field-collected sediments are toxic and may include controls, reference sediments, and test sediments. Controls are used to evaluate the acceptability of the test (see 13.3, 14.3, Annex A1 to Annex A7) and might include a control sediment, a formulated sediment (Section 7.2), a sand substrate (for *C. tentans*; see 13.2, A7.2), or water-only exposures (for *H. azteca*; Section A6.3.7.8). Testing a reference sediment provides a site-specific basis for evaluating toxicity of the test sediments. Comparisons of test sediments to multiple reference or control sediments representative of the physical characteristics of the test sediment (i.e., grain size, organic carbon) may be useful in these evaluations. A summary of field sampling design is presented by Green (**62**). See Section 15 for additional guidance on experimental design and statistics.

4.2.2.2 If the purpose of the study is to conduct a reconnaissance field survey to identify sites for further investigation, the experimental design might include only one sample from each site to allow for sampling a larger area. The lack of replication at a site usually precludes statistical comparisons (for example, analysis of variance (ANOVA)), but these surveys can be used to identify sites for further study or may be evaluated using regression techniques.

4.2.2.3 In other instances, the purpose of the study might be to conduct a quantitative sediment survey of chemistry and toxicity to determine statistically significant differences between effects among control and test sediments from several sites. The number of replicates/site should be based on the need for sensitivity or power (see Section 15). In a quantitative survey, field replicates (separate samples from different grabs collected at the same site) would need to be taken at each site. Chemical and physical characterizations of each of these grabs would be required for each of these field replicates used in sediment testing. Separate subsamples might be used to determine within-sample variability or for comparisons of test procedures (for example, comparative sensitivity among test organisms), but these subsamples cannot be considered to be true field replicates for statistical comparisons among sites.

4.2.2.4 Sediments often exhibit high spatial and temporal variability (**63**). Therefore, replicate samples may need to be collected to determine variance in sediment characteristics. Sediment should be collected with as little disruption as possible; however, subsampling, compositing, or homogenization of sediment samples may be required for some experimental designs.

4.2.2.5 Site locations might be distributed along a known pollution gradient, in relation to the boundary of a disposal site, or at sites identified as being contaminated in a reconnaissance survey. Comparisons can be made in both space and time. In pre-dredging studies, a sampling design can be prepared to assess the contamination of samples representative of the project area to be dredged. Such a design should include subsampling cores taken to the project depth.

4.2.2.6 The primary focus of the physical and experimental test design and statistical analysis of the data, is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned (Guide E 1241). Because overlying water or air cannot flow from one test chamber to another the test chamber is the experimental unit. The experimental unit is defined as the smallest physical entity to which treatments can be independently assigned and to which air and water exchange between test chambers are kept to a minimum. Because of factors that might affect results within test chambers and results of a test, all test chambers should be treated as similarly as possible. Treatments should be randomly assigned to individual test chamber locations. Assignment of test organisms to test chambers should be impartial (see Guide E 729). As the number of test chambers/treatment increases, the number of degrees of freedom increases, and, therefore, the width of the confidence interval on a point estimate, such as an LC50, decreases, and the power of a significance test increases (see Section 15).

5. Significance and Use

5.1 *General*:

5.1.1 Sediment provides habitat for many aquatic organisms and is a major repository for many of the more persistent chemicals that are introduced into surface waters. In the aquatic environment, most anthropogenic chemicals and waste materials including toxic organic and inorganic chemicals eventually accumulate in sediment. Mounting evidences exists of environmental degradation in areas where USEPA Water Quality Criteria (WQC; (**64**)) are not exceeded, yet organisms in or near sediments are adversely affected (**65**). The WQC were developed to protect organisms in the water column and were not directed toward protecting organisms in sediment. Concentrations of contaminants in sediment may be several orders of magnitude higher than in the overlying water; however, bulk sediment concentrations have not been strongly correlated to bioavailability (**66**). Partitioning or sorption of a compound between water and sediment may depend on many factors including: aqueous solubility, pH, redox, affinity for sediment organic carbon and dissolved organic carbon, grain size of the sediment, sediment mineral constituents (oxides of iron, manganese, and aluminum), and the quantity of acid volatile sulfides in sediment (**39, 40**). Although certain chemicals are highly sorbed to sediment, these compounds may still be available to the biota. Chemicals in sediments may be directly toxic to aquatic life or can be a source of chemicals for bioaccumulation in the food chain.

5.1.2 The objective of a sediment test is to determine whether chemicals in sediment are harmful to or are bioaccumulated by benthic organisms. The tests can be used to measure interactive toxic effects of complex chemical mixtures in sediment. Furthermore, knowledge of specific pathways of interactions among sediments and test organisms is not necessary to conduct the tests (**67**). Sediment tests can be used to: *(1)* determine the relationship between toxic effects and bioavailability, *(2)* investigate interactions among chemicals, *(3)* compare the sensitivities of different organisms, *(4)* determine spatial and temporal distribution of contamination, *(5)* evaluate hazards of dredged material, *(6)* measure toxicity [illegible]

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product licensing or safety testing, *(7)* rank areas for clean up, and *(8)* estimate the effectiveness of remediation or management practices.

5.1.3 A variety of methods have been developed for assessing the toxicity of chemicals in sediments using amphipods, midges, polychaetes, oligochaetes, mayflies, or cladocerans (Section 13 and 14; Annex A1 to Annex A5; **(1)**, **(3)**, **(355)**, **(389)**. Several endpoints are suggested in these methods to measure potential effects of contaminants in sediment including survival, growth, behavior, or reproduction; however, survival of test organisms in 10-day exposures is the endpoint most commonly reported. These short-term exposures which only measure effects on survival can be used to identify high levels of contamination in sediments, but may not be able to identify moderate levels of contamination in sediments (USEPA **(1)**; Sibley et al., **(53)**; Sibley et al., **(54)**; Sibley et al., **(68)**; Benoit et al., **(69)**; Ingersoll et al., **(55)**). Sublethal endpoints in sediment tests might also prove to be better estimates of responses of benthic communities to contaminants in the field **(17)**. The previous version of this standard (Test Method E 1706-95b) described 10-day toxicity tests with the amphipod *Hyalella azteca* and midge *Chironomus tentans* (see Section 13 and 14). This version of the standard now outlines approaches for evaluating sublethal endpoints in longer-term sediment exposures with these two species (Annex A6 and Annex A7).

5.1.3.1 The decision to conduct short-term or long-term toxicity tests depends on the goal of the assessment. In some instances, sufficient information may be gained by measuring sublethal endpoints in 10-day tests. In other instances, the 10-day tests could be used to screen samples for toxicity before long-term tests are conducted. While the long-term tests are needed to determine direct effects on reproduction, measurement of growth in these toxicity tests may serve as an indirect estimate of reproductive effects of contaminants associated with sediments (A6.4.5 and A7.4.6.2). Additional studies are ongoing to more thoroughly evaluate the relative sensitivity between lethal and sublethal endpoints measured in 10-day tests (Sections 13 and 14) and between sublethal endpoints measured in the long-term tests. Results of these studies and additional applications of the methods described in Annex A6 and Annex A7 will provide data that can be used to assist in determining where application of long-term tests will be most appropriate.

5.1.3.2 Use of sublethal endpoints for assessment of contaminant risk is not unique to toxicity testing with sediments. Numerous regulatory programs require the use of sublethal endpoints in the decision-making process (Pittinger and Adams **(400)**) including: (1) Water Quality Criteria (and State Standards); (2) National Pollution Discharge Elimination System (NPDES) effluent monitoring (including chemical-specific limits and sublethal endpoints in toxicity tests); (3) Federal Insecticide, Rodenticide and Fungicide Act (FIFRA) and the Toxic Substances Control Act (TSCA, tiered assessment includes several sublethal endpoints with fish and aquatic invertebrates); (4) Superfund (Comprehensive Environmental Responses, Compensation and Liability Act; CERCLA); (5) Organization of Economic Cooperation and Development (OECD, sublethal toxicity testing with fish and invertebrates); (6) European Economic Community (EC, sublethal toxicity testing with fish and invertebrates); and (7) the Paris Commission (behavioral endpoints).

5.1.4 Results of toxicity tests on sediments spiked at different concentrations of chemicals can be used to establish cause and effect relationships between chemicals and biological responses. Results of toxicity tests with test materials spiked into sediments at different concentrations may be reported in terms of an LC50 (median lethal concentration), an EC50 (median effect concentration), an IC50 (inhibition concentration), or as a NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration). However, spiked sediment may not be representative of chemicals associated with sediment in the field. Mixing time **(70)**, aging **(35, 71, 72)**, and the chemical form of the material can affect responses of test organisms in spiked sediment tests.

5.1.5 Evaluating effect concentrations for chemicals in sediment requires knowledge of factors controlling their bioavailability. Similar concentrations of a chemical in units of mass of chemical per mass of sediment dry weight often exhibit a range in toxicity in different sediments **(38, 39)**. Effect concentrations of chemicals in sediment have been correlated to interstitial water concentrations, and effect concentrations in interstitial water are often similar to effect concentrations in water-only exposures. The bioavailability of nonionic organic compounds in sediment is often inversely correlated with the organic carbon concentration. Whatever the route of exposure, these correlations of effect concentrations to interstitial water concentrations indicate that predicted or measured concentrations in interstitial water can be used to quantify the exposure concentration to an organism. Therefore, information on partitioning of chemicals between solid and liquid phases of sediment is useful for establishing effect concentrations **(39)**.

5.1.6 Field surveys can be designed to provide either a qualitative reconnaissance of the distribution of sediment contamination or a quantitative statistical comparison of contamination among sites.

5.1.7 Surveys of sediment toxicity are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlations may be improved and sampling costs may be reduced if subsamples are taken simultaneously for sediment tests, chemical analyses, and benthic community structure.

5.1.8 Table 3 lists several approaches the USEPA has considered for the assessment of sediment quality **(73)**. These approaches include: *(1)* equilibrium partitioning, *(2)* tissue residues, *(3)* interstitial water toxicity, *(4)* whole-sediment toxicity and sediment-spiking tests, *(5)* benthic community structure, *(6)* effect ranges (for example, effect range median, ERM), and *(7)* sediment quality triad (see **(74-77)** for a critique of these methods). The sediment assessment approaches listed in Table 3 can be classified as numeric (for example, equilibrium partitioning), descriptive (for example, whole-sediment toxicity tests), or a combination of numeric and descriptive approaches (for example, ERM, **(78)**. Numeric methods can be used to derive chemical-specific sediment quality guidelines (SQGs). Descriptive methods such as toxicity tests with

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TABLE 3 Sediment Quality Assessment Procedures (Modified from USEPA (78))

Method	Type			Approach
	Numeric	Descriptive	Combination	
Equilibrium Partitioning		*		A sediment quality value for a given contaminant is determined by calculating the sediment concentration of the contaminant that corresponds to an interstitial water concentration equivalent to the USEPA water-quality criterion for the contaminant.
Tissue Residues	*			Safe sediment concentrations of specific chemicals are established by determining the sediment chemical concentration that results in acceptable tissue residues.
Interstitial Water Toxicity	*	*	*	Toxicity of interstitial water is quantified and identification evaluation procedures are applied to identify and quantify chemical components responsible for sediment toxicity.
Benthic Community Structure		*		Environmental degradation is measured by evaluating alterations in benthic community structure.
Whole-sediment Toxicity and Sediment Spiking	*	*	*	Test organisms are exposed to sediments that may contain known or unknown quantities of potentially toxic chemicals. At the end of a specified time period, the response of the test organisms is examined in relation to a specified endpoint. Dose-response relationships can be established by exposing test organisms to sediments that have been spiked with known amounts of chemicals or mixtures of chemicals.
Sediment Quality Triad	*	*	*	Sediment chemical contamination, sediment toxicity, and benthic community structure are measured on the same sediment sample. Correspondence between sediment chemistry, toxicity, and field effects is used to determine sediment concentrations that discriminate conditions of minimal, uncertain, and major biological effects.
Sediment Quality Guidelines	*	*	*	The sediment concentration of contaminants associated with toxic responses measured in laboratory exposures or field assessments (i.e., Apparent Effects Threshold (AET), Effect Range Median (ERM), Probable Effect Level (PEL).

field-collected sediment cannot be used alone to develop numerical SQGs for individual chemicals. Although each approach can be used to make site-specific decisions, no one single approach can adequately address sediment quality. Overall, an integration of several methods using the weight of evidence is the most desirable approach for assessing the effects of contaminants associated with sediment (**79**, **80**, **81**, **82**). Hazard evaluations integrating data from laboratory exposures, chemical analyses, and benthic community assessments (the sediment quality triad) provide strong complementary evidence of the degree of pollution-induced degradation in aquatic communities (**66**, **83**, **84**).

5.2 *Regulatory Applications of Sediment Tests*:

5.2.1 The USEPA has authority under a variety of statutes to manage contaminated sediments (Table 4). USEPA's Contaminated Sediment Management Strategy (**85**, **86**) establishes the following four goals for contaminated sediments and describes actions that the Agency intends to take to accomplish these goals: (1) to prevent further contamination of sediments that may cause unacceptable ecological or human health risks; (2) when practical, to clean up existing sediment contamination that adversely affects the Nation's waterbodies or their uses, or that causes other significant effects on human health or the environment; (3) to ensure that sediment dredging and the disposal of dredged material continue to be managed in an environmentally sound manner; and (4) to develop and consistently apply methodologies for analyzing contaminated sediments. The Agency plans to employ its pollution prevention and source control programs to address the first goal. To accomplish the second goal, USEPA will consider a range of risk management alternatives to reduce the volume and effects of existing contaminated sediments, including *in-situ* containment and contaminated sediment removal. Finally, the Agency is developing tools for use in pollution prevention, source control, remediation, and dredged material management to meet the collective goals. These tools include national inventories of sediment quality and environmental releases of contaminants, numerical assessment guidelines to evaluate contaminant concentrations, and standardized bioassays to evaluate the bioaccumulation and toxicity potential of sediment samples.

TABLE 4 Statutory Needs for Sediment Quality Assessment (Modified from Dickson et al (90) and Southerland et al (85))

Law[A]	Area of need
CERCLA	—Assess need for remedial action with contaminated sediments; assess degree of cleanup required; disposition of sediment
CWA	—NPDES permitting, especially under Best Available Technology (BAT) in water-quality-limited water
	—Section 403(c) criteria for ocean discharges; mandatory additional requirements to protect marine environment
	—Section 301(g) waivers for publically owned treatment works (PTOWS) discharging to marine waters
	—Section 404 permits to dredge and fill activities (administered by the Corps of Engineers)
FIFRA	—Review uses of new and existing chemicals
	—Pesticide labeling and registration
MPRSA	—Permits for ocean dumping
NEPA	—Preparation of environmental impact statements for projects with surface water discharges
TSCA	—Section 5: Pre-manufacture notice reviews for new chemicals
	—Section 4,5,6: Reviews for existing industrial chemicals
RCRA	—Assess suitability (and permit) on-land disposal or beneficial use of contaminated sediments considered "hazardous"

[A] CERCLA Comprehensive Environmental Response, Compensation and Liability Act ("Superfund")
CWA Clean Water Act
FIFRA Federal Insecticide, Fungicide, and Rodenticide Act
MPRSA Marine Protection, Resources and Sanctuary Act
NEPA National Environmental Policy Act
TSCA Toxic Substances Control Act
RCRA Resource Conservation and Recovery Act

5.2.2 The Clean Water Act (CWA) is the single most important law dealing with environmental quality of surface waters in the United States. The objective of the CWA is to restore and maintain the chemical, physical, and biological

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integrity of the nation's waters (CWA, Section 101). Federal and state monitoring programs traditionally have focused on evaluating water column problems caused by point source dischargers. Findings in the National Sediment Quality Survey, volume I of the first biennial report to Congress on sediment quality in the U.S., indicate that this focus needs to be expanded to include sediment quality impacts (Section 1.1.2 and (**87**).

5.2.3 The Office of Water (OW), the Office of Prevention, Pesticides, and Toxic Substances (OPPTS), the Office of Solid Waste (OSW), and the Office of Emergency and Remedial Response (OERR) are all committed to the principle of consistent tiered testing described in the Contaminated Sediment Management Strategy (USEPA, (**86**)). Agency-wide consistent testing is desirable because all USEPA programs will use standard methods to evaluate health risk and produce comparable data. It will also provide the basis for uniform cross-program decision-making within the USEPA. Each program will, however, retain the flexibility of deciding whether identified risks would trigger regulatory actions.

5.2.4 Tiered testing refers to a structured, hierarchial procedure for determining data needs relative to decision-making that consists of a series of tiers, or levels, of investigative intensity. Typically, increasing tiers in a tiered testing framework involve increased information and decreased uncertainty (USEPA, (**86**)). Each EPA program office intends to develop guidance for interpreting the tests conducted within the tiered framework and to explain how information within each tier would trigger regulatory action. Depending on statutory and regulatory requirements, the program specific guidance will describe decisions based on a weight of evidence approach, a pass-fail approach, or comparison to a reference site. The following two approaches are currently being used by USEPA: (1) the Office of Water-U.S. Army Corps of Engineers dredged material testing framework and (2) the OPPTS ecological risk assessment tiered testing framework. USEPA-USACE (**107**) describes the dredged material testing framework and Smrchek and Zeeman (**401**) summarizes the OPPTS testing framework. A tiered testing framework has not yet been chosen for agency-wide use, but some of the components have been identified to be standardized. These components are toxicity tests, bioaccumulation tests, chemical criteria, and other measurements that may have ecological significance including benthic community structure, colonization rate, and *in situ* testing within a mesocosm (**73**).

5.3 *Performance-based criteria*:

5.3.1 The USEPA's Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards (**88**). Performance-based methods were defined by EMMC as a monitoring approach which permits the use of appropriate methods that meet preestablished demonstrated performance standards (see 11.2).

5.3.2 The USEPA Office of Water, Office of Science and Technology, and Office of Research and Development held a workshop to provide an opportunity for experts in the field of sediment toxicology and staff from USEPA's Regional and Headquarters Program offices to discuss the development of standard freshwater and marine sediment testing procedures (**73, 89**). Workgroup participants arrived at a consensus on several culturing and testing methods. In developing guidance for culturing test organisms to be included in the USEPA's methods manual for sediment tests, it was agreed that no one method should be required to culture organisms. However, the consensus at the workshop was that success of a test depends on the health of the cultures. Therefore, having healthy test organisms of known quality and age for testing was determined to be the key consideration relative to culturing methods. A performance-based criteria approach was selected in USEPA (**1**) as the preferred method through which individual laboratories could use unique culturing methods rather than requiring use of one culturing method.

5.3.3 This standard recommends the use of performance-based criteria to allow each laboratory to optimize culture methods and minimize effects of test organism health on the reliability and comparability of test results. See 13.1.2 and 14.1.2 and Annex A1 to Annex A7 for a listing of performance criteria for culturing and testing.

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 Because of the heterogeneity of natural sediments, extrapolation from laboratory studies to the field can sometimes be difficult (Table 5; (**66**)). Sediment collection, handling, and storage may alter bioavailability and concentration by changing the physical, chemical, or biological characteristics of the sediment. Maintaining the integrity of a field-collected sediment during removal, transport, mixing, storage, and testing is extremely difficult and may complicate the interpretation of effects. See (**61**) and Guide 1391.

6.1.3 Depletion of aqueous and sediment-sorbed chemicals resulting from uptake by an organism or test chamber may also influence availability. In most cases, the organism is a minor sink for chemicals relative to the sediment. However, within the burrow of an organism, sediment desorption kinetics may limit uptake rates. Within minutes to hours, a major portion of the total chemical may be inaccessible to the organisms because of depletion of available residues. The desorption of a particular compound from sediment may range from easily reversible (labile; within minutes) to irreversible (non-labile; within days or months (**91**)). Interparticle diffusion or advection and the quality and quantity of sediment organic carbon can also affect sorption kinetics.

6.1.4 Testing sediments at temperatures different from the field might affect contaminant solubility, partitioning coefficients, or other physical and chemical characteristics. Interaction between sediment and overlying water and the ratio of sediment to overlying water may influence bioavailability (**79**).

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TABLE 5 Advantages and Disadvantages for Use of Sediment Tests (Modified from Swartz (110))

Advantages
—Measure bioavailable fraction of contaminant(s).
—Provide a direct measure of benthic effects, 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; ASTM standard guides are available.
—Measure unique information relative to chemical analyses or benthic 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 benthos populations.
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.

6.1.5 Results of sediment tests can be used to predict effects that may occur with aquatic organisms in the field as a result of exposure under comparable conditions. However, motile organisms might avoid exposure in the field. Photoinduced toxicity may be important for some compounds associated with sediment (for example, polycyclic aromatic hydrocarbons (PAHs) (**92**)). However, lighting typically used to conduct laboratory tests does not include the appropriate spectrum of ultraviolet radiation to photoactivate compounds (**93**, **94**) and thus laboratory tests may not account for toxicity expressed by this mode of action.

6.1.6 Natural physico-chemical characteristics such as sediment texture may influence the response of test organisms (**56**). The physico-chemical characteristics of test sediment need to be within the tolerance limits of the test organism. Ideally, the limits of the test organism should be determined in advance; however, control samples reflecting differences in factors such as grain size and organic carbon can be evaluated if the limits are exceeded in the test sediment (see 12.1). The effects of sediment characteristics can also be addressed with regression equations (**56**, **57**). The use of formulated sediment can also be used to evaluate physico-chemical characteristics of sediment on test organisms (**58**, **59**).

6.1.7 Indigenous organisms may be present in field-collected sediments. An abundance of the same organism or organisms taxonomically similar to the test organism in the sediment sample may make interpretation of treatment effects difficult. For example, growth of amphipods, midges, or mayflies may be reduced if high numbers of oligochaetes are in a sediment sample (**95**). Previous investigators have inhibited the biological activity of sediment with sieving, heat, mercuric chloride, antibiotics, or gamma irradiation (Guide E 1391, (**96**)). However, further research is needed to determine effects on contaminant bioavailability or other modifications of sediments from treatments such as those used to remove or destroy indigenous organisms.

6.1.8 The route of exposure may be uncertain and data from sediment tests may be difficult to interpret if factors controlling the bioavailability of chemicals in sediment are unknown. Bulk-sediment chemical concentrations may be normalized to factors other than dry weight. For example, concentrations of nonionic organic compounds might be normalized to sediment organic-carbon content (**78**) and certain metals normalized to acid volatile sulfides (**38**). Even with the appropriate normalizing factors, determination of toxic effects from ingestion of sediment or from dissolved chemicals in the interstitial water can still be difficult (**97**).

6.1.9 The addition of food, water, or solvents to the test chambers might obscure the bioavailability of chemicals in sediment or might provide a substrate for bacterial or fungal growth. Without addition of food, the test organisms may starve during exposures (**57**, **98**). However, the addition of the food may alter the availability of the chemicals in the sediment (**34**, **99**) depending on the amount of food added, its composition (for example, total organic carbon (TOC)), and the chemical(s) of interest.

6.1.10 Laboratory sediment testing with field-collected sediments may be useful in estimating cumulative effects and interactions of multiple contaminants in a sample. Tests with field samples usually cannot discriminate between effects of individual chemicals. Many sediment samples contain a complex matrix of inorganic and organic chemicals with many unidentified compounds. The use of Toxicity Identification Evaluations (TIE) procedures including sediment tests with spiked chemicals may provide evidence of causal relationships and can be applied to many chemicals of concern (**9**). Laboratory studies that test single compounds spiked into the sediment can be used to determine more directly the specific chemicals causing a toxic response (**100**).

6.1.11 Sediment spiking can also be used to investigate additive, antagonistic, or synergistic effects of specific chemical mixtures in a sediment sample (**100**). However, spiked sediment may not be representative of contaminated sediment in the field. Mixing time (**63**) and aging (**35**, **71**, **72**) of spiked sediment can affect responses of organisms.

6.1.12 Most assessments of contaminated sediment rely on acute-lethality testing methods (for example, ≤10 days; (**105-107**) . Acute-lethality tests are useful in identifying "hot spots" of sediment contamination, but may not be sensitive enough to evaluate moderately contaminated areas. Sediment quality assessments using sublethal responses of benthic organisms such as effects on growth and reproduction have been used to successfully evaluate moderately contaminated areas (**108**, **17**, **55**), Annex A6 and Annex A7.

6.1.13 Despite the interferences previously listed, existing sediment testing methods that include measurement of sublethal endpoints may be used to provide a rapid and direct measure of effects of contaminants on benthic communities.

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(e.g., Canfield et al. (**45**)). Laboratory tests with field-collected sediment can also be used to determine temporal, horizontal, or vertical distribution of contaminants in sediment. Most tests can be completed within two to four weeks. Legal and scientific precedence exist for use of sediment tests in regulatory decision making (for example, (**109, 110**)). Furthermore, sediment tests with complex contaminant mixtures are important tools for making decisions about the extent of remedial action for contaminated aquatic sites and for evaluating the success of remediation activities.

6.2 *Species-Specific Interferences*—Interferences of tests for each species are described in Sections 13 and 14 and in Annex A1 to Annex A7.

7. Reagents and Materials

7.1 *Water*:

7.1.1 *Requirements*:

7.1.1.1 Water used to test and culture organisms should be uniform in quality. Acceptable water should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (for example, discoloration, unusual behavior). If problems are observed in the culturing or testing of organisms, it is desirable to evaluate the characteristics of the water. See USEPA (**2**) and Guide E 1367 for a recommended list of chemical analyses of the water supply.

7.1.1.2 When deionized water is required, the water-deionizing system should provide sufficient quantity of at least 1 MΩ of water. If large quantities of high-quality deionized water are needed, it may be advisable to supply the laboratory-grade water deionizer with preconditioned water from a mixed-bed water treatment system. Some investigators have observed that holding reconstituted water prepared from deionized water for several days before use in sediment tests may be improve performance of test organisms (C. Hickey, National Institute of Water and Atmospheric Research, Hamilton, New Zealand, personal communication).

7.1.2 *Source*:

7.1.2.1 A natural water is considered to be of uniform quality if monthly ranges of the hardness, alkalinity, and specific conductance are <10 % of their respective averages and if the monthly range of pH is <0.4. Natural waters should be obtained from an uncontaminated well or spring, if possible, or from a surface-water source. If surface water is used, the intake should be positioned to: *(1)* minimize fluctuations in quality and contamination, *(2)* maximize the concentration of dissolved oxygen, and *(3)* ensure low concentrations of sulfide and iron. Municipal-water supplies may be variable and may contain unacceptably high concentrations of materials such as copper, lead, zinc, fluoride, chlorine, or chloramines. Chlorinated water should not be used for culturing or testing because residual chlorine and chlorine-produced oxidants are toxic to many aquatic organisms. Dechlorinated water should only be used as a last resort since dechlorination is often incomplete (Guide E 1241, (**2**)).

7.1.2.2 For site-specific investigations, it is desirable to have the water-quality characteristics of the overlying water as similar as possible to the site water. For certain applications the experimental design might require use of water from the site from which sediment is collected. When distilled water was added to sediment, contaminant and organic carbon distributed on smaller sediment particles (perhaps resulting from disaggregation of particles). Therefore, it may be advisable to conduct sediment tests with water representative of the site of concern (**1**).

7.1.2.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter and flow controls or passed through a filter with a pore size of ≤0.45 µm.

7.1.2.4 Water might need aeration using air stones, surface aerators, or column aerators. Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. Excessive aeration may reduce hardness and alkalinity of hard water. The concentration of dissolved oxygen in source water should be between 90 to 100 % saturation to help ensure that dissolved oxygen concentrations are acceptable in test chambers. It may be desirable to aerate dechlorinated water before use (for example, 3 days).

7.1.3 *Reconstituted Water*:

7.1.3.1 Ideally, reconstituted water is prepared by adding specified amounts of reagent-grade[7] chemicals to high-purity distilled or deionized water (Guide E 729, (**2**)). Problems have been observed with the use of reconstituted water in long-term exposures with *H. azteca* (Section 7.1.3.4.3). In some applications, acceptable high-purity water can be prepared using deionization, distillation, or reverse-osmosis units (see 9.3, (**2**)). Test water can also be prepared by diluting natural water with deionized water (**17**) or by adding salts to relatively dilute natural waters.

7.1.3.2 Deionized water should be obtained from a system capable of producing at least 1 MΩ water.

7.1.3.3 Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured on each batch of reconstituted water. The reconstituted water should be aerated before use to adjust pH and dissolved oxygen to the acceptable ranges (for example, see 7.1.3.4). USEPA (**2**) recommends using a batch of reconstituted water for less than two weeks.

7.1.3.4 *Reconstituted Fresh Water*—To prepare 100 L of reconstituted fresh water described in Smith et al. (**111**), use the reagent grade chemicals as follows:

(1) Place about 75 L of deionized water in a properly cleaned container.

(2) Add 5 g of $CaSO_4$ and 5 g of $CaCl_2$ to a 2-L aliquot of deionized water and mix (for example, on a stir plate) for 30 min.

(3) Add 3 g of $MgSO_4$, 9.6 g $NaHCO_3$, and 0.4 g KCl to a second 2-L aliquot of deionized water and mix on a stir plate for 30 min or until the salts dissolve.

[7] *Reagent Chemicals, American Chemical Society Specifications,* American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

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(4) Pour the two 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.

(5) Aerate the mixture for at least 24 h before use.

(6) The water quality of the reconstituted water (reformulated moderately hard reconstituted water described by Smith et al. (**111**) and in USEPA (**1**)) should be about: hardness, 90 to 100 mg/L as $CaCO_3$, alkalinity 50 to 70 mg/L as $CaCO_3$, conductivity 330 to 360 µS/cm, and pH 7.8 to 8.2.

(7) McNulty et al. (**98**)and Kemble et al. (**112**) ,(**60**)observed poor survival of *H. azteca* in tests conducted 14 to 28 days using a variety of reconstituted waters including the reconstituted water described by Smith et al. (**111**) in 7.1.3.4. Borgmann (**113**)described a reconstituted water that was used successfully to maintain *H. azteca* in culture; however, some laboratories have not had success with reproduction of the *H. azteca* when using this reconstituted water in the 42-day test (T.J. Norberg-King, USEPA, Duluth, MN, personal communication). Research is ongoing to develop additional types of reconstituted waters suitable for *H. azteca*. Until an acceptable reconstituted water has been developed for long-term exposures with *H. azteca*, a natural water demonstrated to support adequate survival, growth, and reproduction of amphipods is recommended for use in long-term *H. azteca* exposures (Annex A6.2; (**55, 112, 60**)).

7.1.3.5 *Synthetic Seawater*—Reconstituted salt water can be prepared by adding commercial sea salts to deionized water. A synthetic seawater formulation can be prepared with reagent grade chemicals which can be diluted with deionized water to the desired salinity (**114**). Ingersoll et al (**115**)describes procedures for culturing *H. azteca* at salinities up to 15 ppt. Reconstituted salt water was prepared by adding commercial salts to a 25:75 (v/v) mixture of freshwater (hardness 283 mg/L as $CaCO_3$) and deionized water that was held at least two weeks before use. Synthetic seawater was conditioned by adding 6.2 mL of nitrifying bacteria No. 9[8] (*Nitromonas sp.* and *Nitrobacter sp.*) to each liter of water. The cultures were maintained by using renewal of water (25 % of the culture water was replaced weekly). *Hyalella azteca* have been used to evaluate the toxicity of estuarine sediments up to 15 ppt salinity in 10-day exposures (**47, 81, 116-118,**).

7.2 *Formulated Sediment*:

7.2.1 *General Requirements*:

7.2.1.1 Formulated sediments are mixtures of materials which mimic the physical components of natural sediments. Formulated sediments have not been routinely applied to evaluate sediment contamination. A primary use of formulated sediment could be as a control sediment. Formulated sediments allow for standardization of sediment testing or provide a basis for conducting sediment research. Formulated sediment provides a basis by which any testing program can assess the acceptability of their procedures and facilities. In addition, formulated sediment provides a consistent measure evaluating performance-based criteria necessary for test acceptability. The use of formulated sediment eliminates interferences caused by the presence of indigenous organisms. Spiking formulated sediments with specific chemicals would reduce variation in sediment physico-chemical characteristics and would provide a consistent method for evaluating the fate of chemicals in sediment. See (**61**) and Guide E 1391 for additional detail regarding uses of formulated sediment.

7.2.1.2 Ideally, a formulated sediment should: (*1*) support the survival, growth, or reproduction of a variety of benthic invertebrates, (*2*) provide consistent acceptable biological endpoints for a variety of species, and (*3*) be composed of materials that have consistent characteristics. Consistent material characteristics include: (*1*) consistency of materials from batch to batch, (*2*) contaminant concentrations below concentrations of concern, and (*3*) availability to all individuals and facilities (**60**).

7.2.1.3 Physico-chemical characteristics which might be considered when evaluating the appropriateness of a formulated sediment include: percent sand, percent clay, percent silt, organic carbon content, cation exchange capacity (CEC), oxidation reduction potential (redox), pH, and carbon: nitrogen:phosphorus ratios.

7.2.2 *Sources of Materials*:

7.2.2.1 A variety of methods describe procedures for making formulated sediments. These procedures often use similar constituents; however, they often include either a component or a formulation step which would result in variation from test facility to test facility. In addition, most of the procedures have not been subjected to standardization and consensus approval or round-robin (ring) testing. The procedure outlined below by Kemble et al. (**60**) was evaluated in round-robin testing with *Hyalella azteca* and *Chironomus tentans* (Section 17.6; USEPA (**1**)).

7.2.2.2 Most formulated sediments include sand and clay/silt which meet certain specifications; however, they may be quite different. For example, three sources of clay and silt include Attagel® 50, ASP® 400, and ASP® 400P.[9] Table 6 summarizes the characteristics of these materials. The percentage of clay ranges from 56.5 to 88.5 and silt ranges from 11.5 to 43.5. These characteristics should be evaluated when considering the materials to use in a formulated sediment.

7.2.2.3 A critical component of formulated sediment is the source of organic compound. Many procedures have used peat as the source of organic carbon. Other sources of organic carbon have been evaluated including: humus, potting soil, maple leaves, composted cow manure, rabbit chow, cereal

[8] Nitrifying bacteria (*Nitromonas sp.* and *Nitrobacter sp.*) such as Frit-zyme® No. 9, available from Fritz Chemical Company, Dallas, TX.

[9] Sources of Kaolinite such as Attagel® 50, ASP® 400, ASP® 400P®, ASP® 600, and ASP® 900 are available from Englehard Corporation, Edison, NJ or Product No. 33059 available from BDH Chemical Ltd, Poole, England.

TABLE 6 Characteristics of 3 Different Sources of Clays and Silts Used in Formulated Sediments[9](See Table 8 for a List of Suppliers)

Characteristic	Attagel® 50	ASP® 400	ASP® 400P
% Sand	0.00	0.01	0.00
% Clay	88.50	68.49	56.50
% Silt	11.50	31.50	43.50
Soil Class	Clay	Clay	Silty clay

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leaves,[10] *Chlorella*, trout chow, fish food flakes[11] and fish food flakes.[12], and alpha cellulose. Only peat, humus, potting soil, composted cow manure, and alpha cellulose have been used successfully without fouling the overlying water in sediment testing (**60**). The other sources of organic carbon listed in Table 7 caused dissolved oxygen concentrations to fall to unacceptable levels (**1, 60**). Kemble (**60**) reported that conditioning of formulated sediment was not necessary when alpha cellulose was used as a source of organic carbon to prepare sediment for use as a negative control. In addition, alpha cellulose is a consistent source of organic carbon, low in contaminant concentrations of concern, and is a relatively biologically-inactive source of organic carbon. There are three forms of cellulose (alpha, beta, and gamma) that differ in their degree of polymerization, alpha cellulose has the highest degree of polymerization and is the chief constituent of paper pulp. The beta and gamma forms have much lower degree of polymerization and are known as hemicellulose. Hence, the alpha cellulose would not serve as food source for test organisms compared to other sources of organic carbon, but would serve as a organic carbon constituent for sediment (i.e., texture or a partitioning compartment for contaminants). The use of alpha cellulose as a source of organic carbon for sediment-spiking studies has not been adequately evaluated. A recent study conducted by J. Besser (USGS, Columbia, MO, unpublished data) indicate that use of alpha cellulose as a source of organic carbon in 21-day studies resulted in some generation of sulfide in the pore water (which may affect the bioavailability of metals spiked in sediment).

7.2.2.4 An important consideration in the selection of an organic carbon source may be the ratio of carbon:nitrogen-:phosphorus. As demonstrated in Table 7, percentage carbon ranged from 30 to 47, percentage nitrogen ranged from 0.7 to 45 mg/g, and percentage phosphorus ranged from below detection to 11 µg/g for several different carbon sources. These characteristics should be evaluated when considering the materials to use in a formulated sediment.

7.2.3 *Procedure*:

[10] Ground cereal leaves, such as Cerophyl, available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, has been found suitable for this purpose.

[11] Fish food flakes such as Tetrafin, available from many pet food distributors, have been found suitable for this purpose.

[12] Fish food flakes such as Tetramin, available from many pet food distributors, have been found suitable for this purpose.

TABLE 7 Carbon, Nitrogen, Phosphorus Levels for Various Sources of Organic Carbon (1)

Organic Carbon Source	Carbon, %	Nitrogen, mg/g	Phosphorus, µg/g
Peat	47	4	0.4
Maple leaves 1	42	6	1.3
Maple leaves 2	47	3	1.7
Cow manure	30	11	8.2
Rabbit chow	40	18	0.2
Humic acid	40	3	...
Cereal leaves[10]	47	4	0.4
Chlorella	40	41	5.7
Trout chow	43	36	11
Fish food flakes[12]	37	45	9.6
Fish food flakes[11]36	29	8.6	

7.2.3.1 A summary of procedures that have been used to formulate sediment are listed as follows. Suppliers of various components are listed in Table 8.

(1) Walsh et al (**58**): Wash and sieve sand into three grain sizes: coarse (500 to 1500 µm); medium (250 to 499 µm); and fine (63 to 249 µm); Mystic White No. 85, 45, and 18-New England Silica Inc.; Note: Mystic White sands are no longer available. Kemble et al. (**60**) found White Quartz sand to be an acceptable substitute; Table 8). Obtain clay and silt from Engelhard Corp. Mill peat moss and sieved through a 840-µm screen. Mix constituents dry in the following quantities (by weight): coarse sand (0.6 %); medium sand (8.7 %); fine sand (69.2 %); silt (10.2 %); clay (6.4 %); and organic matter (4.9 %).

(2) Harrahy and Clements (**119**): Rinse peat moss then soak for 5 days in deionized water renewing water daily. Acclimate for 5 days, remove all water and spread out to dry. Grind moss and sieve using the following sieve sizes: 1.18 mm (discard these particles); 1.00 mm (average size 1.09 mm); 0.85 mm (average size 0.925); 0.60 (average size 0.725); 0.425 mm (average size 0.5125 mm); retainer (average size 0.2125 mm). Use a mixture of sizes that provides an average particle size of 840 µm. Wash medium quartz sand and dry. Clay and silt are obtained using ASP® 400 (Englehard Corp).[9] Dry mixed constituents in the following quantities: sand (850 g); silt and clay (150 g); dolomite (0.5 g); sphagnum moss (22 g); and humic acid (0.10 g). Mix sediment for an hour on a rolling mill and stored dry until ready for use.

(3) Hanes et al (**120**)**:** Sieve sand and retain two particle sizes (90 to 180 µm and 180 to 250 µm) which are mixed in a ratio of 2:1. Dry potting soil for 24 h at room temperature and sieved through a 1-mm screen. Obtain clay as a commercially available sculptors clay. Determine percent moisture of clay and soil after drying for 24 h at 60 to 100°C (correct for percent moisture when mixing materials). Mix constituents by weight in the following ratios: sand mixture (42 %); clay (42 %); and soil (16 %). Autoclave after mixing, autoclave in a foil-covered

TABLE 8 Sources of Various Components Used in Formulated Sediments

Component	Sources
Sand	•White Quartz sand #1 dry, #2, #3—New England Silica, Inc., South Windsor, CT (Note: Mystic White sands are no longer available. Kemble et al. **(60)** found White Quartz sand to be an acceptable substitute.
	•Product No. 33094, BDH Chemical, Ltd., Poole, England
Kaolinite	•ASP 400, ASP 400P, ASP 600, ASP 900—Englehard Corporation, Edison, NJ
	•Product No. 33059, BDH Chemical, Ltd., Poole, England
Montmorillonite	•W.D. Johns, Source Clays, University of Missouri, Columbia, MO
Clay	•Lewiscraft Sculptor's Clay, available in hobby and artist supply stores
Humus	•Sims Bark Co., Inc., Tuscumbia, AL
Alpha cellulose	•Sigma Co., St. Louis, MO
Peat	•D.L. Browning Co., Mather, WI
	•Joseph Bentley, Ltd., Barrow-on-Humber, South Humberside, England
	•Mellinger's, North Lima, OH
Potting soil	•Zehr's No Name Potting Soil, Mississauga, Ontario
Humic acid	•Aldrich Chemical Co., Milwaukee, WI
Cow manure	•A.H. Hoffman, Inc., Landisville, PA
Dolomite	•Ward's Natural Science Establishment, Inc., Rochester, NY

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container for 20 min. Mixture can be stored indefinitely if kept covered after autoclaving.

(4) Naylor et al (**121**): Acid wash sand and sieve to obtain a 40 to 100-mm size. Obtain clay as kaolin light. Grind peat moss and sieve using a 2-mm screen (peat moss that is allowed to dry out will not rehydrate and will float on the water surface). Adjust for the use of moist peat moss by determining moisture content (dry 5 samples of peat at 60°C until constant weight is achieved). Mix constituents by weight in the following percentages: sand (69 %); kaolin (20 %); peat (10 %; adjust for moisture content); and $CaCO_3$ (1 %). Mix for 2 h in a soil shaker and store in sealed containers.

(5) Suedel and Rodgers (**59**): Sieve sand to provide three different size fractions: coarse (2.0 to 0.5 mm), medium (0.5 to 0.25 mm), and fine (0.25 to 0.05 mm; Mystic White No. 18 and 90; Note: Mystic White sands are no longer available, Kemble et al. (**60**) found White Quartz sand to be an acceptable substitute; Table 8). Ash silt (ASP® 400),[9] clay (ASP® 600 and 900),[9] montmorillonite clay, and dolomite are ashed at 550°C for 1 h to remove organic matter. Dry humus (70°C) and milled to 2.0 mm. Add dolomite at 1 % of the silt requirement. Age materials for 7 days in flowing water before mixing. Mix constituents to mimic the desired characteristics of the sediment of concern.

(6) Kemble et al. (**60**) describe procedures for making a variety of formulated sediments ranging in grain size and organic carbon. A sediment with 19 % sand and 2 % organic carbon was produced by combining: (1) 219 grams of sand (White Quartz #1 dry), (2) 1242 grams of a silt-clay mixture (ASP 400), (3) 77.3 grams of alpha cellulose, (4) 0.15 grams of humic acid, and (5) 7.5 grams of dolomite (the dolomite is a source of bicarbonate buffering that occurs naturally in soils and sediments). Steps for processing the sand before use include: (1) rinsing sand with gentle mixing in well water (hardness 283 mg/L as $CaCO_3$ alkalinity 255 mg/L as $CaCO_3$, pH 7.8) until the water runs clear, (2) rinsing the sand for 5 min with deionized water, and (3) air drying the sand. Constituents are mixed for 1 h on a rolling mill and stored dry until ready for use (i.e., no conditioning required). When formulated sediments are made with a high silt-clay content, the alkalinity and hardness of the pore water may drop due to cation exchange. Gentle mixing of the formulated sediment with overlying water before use in testing reduces this change in the water quality characteristics of the pore water.

7.3 *Reagents*—Data sheets should be followed for reagents and other chemicals purchased from supply houses. 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. Reagent containers should be dated when received from the supplier, and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

7.4 *Standards*—Appropriate USEPA, APHA, or ASTM standards for chemical and physical analyses should be used when possible. For those measurements for which standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources.

8. Hazards

8.1 *General Precautions*:

8.1.1 Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management and includes: *(1)* the appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program, *(2)* the preparation of a formal, written health and safety plan, which is provided to each laboratory staff member, *(3)* an ongoing training program on laboratory safety, and *(4)* regular safety inspections.

8.1.2 Collection and use of sediments may involve substantial risks to personal safety and health. Chemicals in field-collected sediment may include carcinogens, mutagens, and other potentially toxic compounds. Inasmuch as sediment testing is often started before chemical analyses can be completed, worker contact with sediment needs to be minimized by: *(1)* using gloves, laboratory coats, safety glasses, face shields, and respirators as appropriate, *(2)* manipulating sediments under a ventilated hood or in an enclosed glove box, and *(3)* enclosing and ventilating the exposure system. Personnel collecting sediment samples and conducting tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation because of lack of oxygen or presence of noxious gases.

8.1.3 Before beginning sample collection and laboratory work, personnel should determine that all required safety equipment and materials have been obtained and are in good condition.

8.2 *Safety Equipment*:

8.2.1 *Personal Safety Gear*—Personnel should use safety equipment, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, face shields, hard hats, and safety shoes.

8.2.2 *Laboratory Safety Equipment*—Each laboratory should be provided with safety equipment such as first-aid kits, fire extinguishers, fire blankets, emergency showers, and eye wash stations. Mobile laboratories should be equipped with a telephone to enable personnel to summon help in case of emergency.

8.3 *General Laboratory and Field Operations*:

8.3.1 Special handling and precautionary guidance in Material Safety Data Sheets (MSDS) should be followed for reagents and other chemicals purchased from supply houses.

8.3.2 Work with some sediments may require compliance with rules pertaining to the handling of hazardous materials. Personnel collecting samples and performing tests should not work alone.

8.3.3 It is advisable to wash exposed parts of the body with bactericidal soap and water immediately after collecting or manipulating sediment samples.

8.3.4 Strong acids and volatile organic solvents should be used in a fume hood or under an exhaust canopy over the work area.

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

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

8.3.7 Use of ground-fault systems and leak detectors is strongly recommended to help prevent electrical shocks. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories should not be used. Ground-fault interrupters should be installed in all "wet" laboratories where electrical equipment is used.

8.3.8 All containers should be adequately labeled to indicate their contents.

8.3.9 A clean and well-organized work place contributes to safety and reliable results.

8.4 *Disease Prevention*—Personnel handling samples which are known or suspected to contain human wastes should be immunized against hepatitis B, tetanus, typhoid fever, and polio. Thorough washing of exposed skin with bactericidal soap should follow handling of samples collected from the field.

8.5 *Safety Manuals*— For further guidance on safe practices when handling sediment samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals including (**122**, **123**).

8.6 *Pollution Prevention, Waste Management, and Sample Disposal*—Guidelines for the handling and disposal of hazardous materials should be strictly followed (Guide D 4447). The Federal Government has published regulations for the management of hazardous waste and has given the States the option of either adopting those regulations or developing their own. If States develop their own regulations, they are required to be at least as stringent as the Federal regulations. As a handler of hazardous materials, it is your responsibility to know and comply with the pertinent regulations applicable in the State in which you are operating. Refer to the Bureau of National Affairs Inc. (**124**) for the citations of the Federal requirements.

9. Facilities, Equipment, and Supplies

9.1 *General*—Before a sediment test is conducted in any new test facility, it is desirable to conduct a "non-toxicant" test, in which all test chambers contain a control sediment, and overlying water with no added test material (see 11.14). Survival, growth, or reproduction of the test organisms will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable species-specific control numbers (for example, see 13.1.2). Evaluations may also be made on the magnitude of the within-chamber and between-chamber variance in a test. See 11.14.

9.2 *Facilities*:

9.2.1 The facility must include separate areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding, acclimation, and culture chambers should not be in a room where sediment tests are conducted, where stock solutions or sediments are prepared, or where equipment is cleaned. Test chambers may be placed in a temperature-controlled recirculating water bath or a constant-temperature area. An enclosed test system is desirable to provide ventilation during tests to limit exposure of laboratory personnel to volatile substances.

9.2.2 Light of the quality and illuminance normally obtained in the laboratory is adequate (about 100 to 1000 l× using wide-spectrum fluorescent lights: for example, cool-white or day-light has been used successfully to culture and test organisms). Lux is the unit selected for reporting luminance in this standard. Multiply units of lux by 0.093 to convert to units of footcandles. Multiply units of lux by 6.91×10^{-3} to convert to units of $\mu mol^{-2}\ s^{-1}$(assuming an average wavelength of 550 nm ($\mu mol^{-2}\ s^{-1}$= W m × λ(nm) × 8.36×10^{-3})). Illuminance should be measured at the surface of the water. A uniform photoperiod of 16L:8D can be achieved in the laboratory or in an environmental chamber using automatic timers.

9.2.3 During rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light or pedestrian traffic.

9.2.4 Air used for aeration should be free of oil and fumes. Filters to remove oil, water, and bacteria are desirable. The test facility should be well ventilated and free of fumes. Oil-free air pumps should be used where possible. Particulates can be removed from the air using filters, and oil and other organic vapors can be removed using activated carbon filters (**1**). Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories or sample handling areas is not circulated to culture or testing areas, or that air from testing areas does not contaminate culture areas. Air pressure differentials between areas should not result in a net flow of potentially contaminated air to sensitive areas through open or loosely fitting doors.

9.3 *Equipment and Supplies*:

9.3.1 Equipment and supplies that contact stock solutions, sediments, or overlying water should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and supplies that contact sediment or water should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, high-density polyethylene, polypropylene, polycarbonate, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Concrete and high-density plastic containers may be used for holding, acclimation, and culture chambers, and in the water-supply system. These materials should be washed in detergent, acid-rinsed, and soaked in flowing water for a week or more before use. Cast-iron pipe should not be used in water-supply systems because colloidal iron will be added to the overlying water and strainers will be needed to remove rust particles. Copper, brass, lead, galvanized metal, and natural rubber must not contact overlying water or stock solutions before or during a test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect survival, growth, or reproduction of the test organisms.

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9.3.2 New lots of plastic products should be tested for toxicity before general use by exposing organisms to them under ordinary test conditions.

9.3.3 *General Equipment*:

9.3.3.1 *Environmental Chamber or Equivalent Facility*, with photoperiod and temperature control (20 to 25°C).

9.3.3.2 *Water Purification System*, capable of producing at least 1 MΩ of water (**2**).

9.3.3.3 *Analytical Balance*, capable of accurately weighing to 0.01 mg.

9.3.3.4 *Reference Weights, Class S*, for documenting the performance of the analytical balance(s). The balance(s) should be checked with reference weights that are at the upper and lower ends of the range of the weighings made when the balance is used. A balance should be checked at the beginning of each series of weighings, periodically (such as every tenth weight) during a long series of weighings, and after taking the last weight of a series.

9.3.3.5 *Volumetric Flasks and Graduated Cylinders*—Class A, borosilicate glass or nontoxic plastic laboratory ware, 10 to 1000 mL for making test solutions.

9.3.3.6 *Volumetric Pipets*—Class A, 1 to 100 mL.

9.3.3.7 *Serological Pipets*—1 to 10 mL, graduated.

9.3.3.8 *Pipet Bulbs and Fillers*.

9.3.3.9 *Droppers, and Glass Tubing with Fire-Polished Edges, 4 to 6-mm inside diameter*, for transferring test organisms.

9.3.3.10 *Wash Bottles*, for rinsing small glassware, instrument electrodes and probes.

9.3.3.11 *Glass or Electronic Thermometers*, for measuring water temperature.

9.3.3.12 *National Bureau of Standards (NBS) Certified Thermometer* (see USEPA Method 170.1; (**125**)).

9.3.3.13 *Dissolved Oxygen (DO), pH/Selective Ion, and Specific Conductivity Meters and Probes*, for routine physical and chemical measurements are needed. Unless a test is being conducted to specifically measure the effect of DO or conductivity, a portable field-grade instrument is acceptable.

9.3.3.14 See Table 9 for a list of additional equipment and supplies. Annex A7 outlines equipment needs for conducting long-term exposures with *Chironomus tentans*.

9.3.4 *Water-delivery System*:

9.3.4.1 The water-delivery system used in water-renewal testing can be one of several designs. The system should be capable of delivering water to each replicate test chamber. Mount and Brungs (**126**) diluters have been successfully modified for sediment testing. Other diluter systems have also been useful (**1, 102, 127-130**) . The water-delivery system should be calibrated before the test by determining the flow rate of the overlying water. The general operation of the system should be visually checked daily throughout the length of the test. If necessary, the water-delivery system should be adjusted during the test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10 %.

9.3.4.2 The overlying water can be replaced manually (for example, siphoning); however, manual systems take more time to maintain during a test. In addition, automated systems generally result in less suspension of sediment compared to manual renewal.

9.3.5 *Test Chambers*:

9.3.5.1 Test chambers may be constructed in several ways and of various materials, depending on the experimental design and the contaminants of interest. Clear silicone adhesives, suitable for aquaria, sorb some organic compounds which might be difficult to remove. Therefore, as little adhesive as possible should be in contact with the test material. Extra beads of adhesive should be on the outside of the test chambers rather than on the inside. To leach potentially toxic compounds from the adhesive, all new test chambers constructed using silicone adhesives should be held at least 48 h in overlying water before use in a test.

9.3.5.2 Test chambers for specific tests are described in Sections 13 and 14, and in Annex A1 to Annex A7.

9.3.6 *Cleaning*:

9.3.6.1 All non-disposable sample containers, test chambers, and other equipment that have come in contact with sediment should be washed after use in the manner described as follows to remove surface contaminants.

(1) Soak 15 min in tap water, and scrub with detergent, or clean in an automatic dishwasher.

(2) Rinse twice with tap water.

(3) Carefully rinse once with fresh, dilute (10 %, V:V) hydrochloric or nitric acid to remove scale, metals, and bases. To prepare a 10 % solution of acid, add 10 mL of concentrated acid to 90 mL of deionized water.

(4) Rinse twice with deionized water.

(5) Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy). Hexane might also be used as a solvent for removing non-ionic organic compounds. However, acetone is preferable if only one organic solvent is used to clean equipment.

(6) Rinse three times with deionized water.

9.3.6.2 All test chambers and equipment should be thoroughly rinsed with the dilution water immediately before use in a test.

9.3.6.3 Many organic solvents leave a film that is insoluble in water. A dichromate-sulfuric acid cleaning solution can be used in place of both the organic solvent and the acid (see Guide E 729), but the solution might attack silicone adhesive and leave chromium residues on glass. An alternative to use of dichromate-sulfuric acid could be to heat glassware for 8 h at 450°C.

10. Sample Collection, Storage, Manipulation, and Characterization

10.1 *Collection*:

10.1.1 Before the preparation or collection of sediment, a procedure should be established for the handling of sediments which might contain unknown quantities of toxic chemicals (Section 8).

10.1.2 Sediments are spatially and temporally variable (**63**). Replicate samples should be collected to determine variance in sediment characteristics. Sediment should be collected with as little disruption as possible; however, subsampling, compositing, or homogenization of sediment samples may be necessary

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TABLE 9 Equipment and Supplies for Culturing and Testing for Specific Test Organisms are Listed As: HA = *Hyalella azteca* and CT = *Chironomus tentans*. See Annex A1 to Annex A5 for Supplies and Equipment for the Additional Test Organisms

A. Biological Supplies	Brood stock of test organisms
	Active dry yeast (HA)
	Cereal leaves; HA
	Trout chow (HA)
	Fish food flakes[11](CA)
	Algae (for example, *Selenastrum capricornutum, Chlorella*; CT)
	Diatoms (for example, *Navicula sp.* (HA)
B. Glassware	Culture chambers
	Test chambers (300-mL high-form lipless beaker; HA and CT)
	Juvenile holding beakers (for example, 1 L; HA)
	Crystallizing dishes or beakers (200 to 300-mL; CT)
	Erlenmeyer flasks (250 and 500 mL; CT)
	Larval rearing chambers (for example, 19-L capacity; CT)
	¼-in. glass tubing (for aspirating flask; CT)
	Wide-bore pipets (4 to 6-mm inside diameter)
	Glass disposable pipets
	Burettes (for hardness and alkalinity determinations)
	Graduated cylinders (assorted sizes, 10 mL to 2 L)
C. Instruments and Equipment	Dissecting microscope
	Stainless steel sieves (for example, U.S. Standard No. 25, 30, 35, 40, 50 mesh)
	Delivery system for overlying water
	Photoperiod timers
	Light meter
	Temperature controllers
	Thermometer
	Continuous recording thermometers
	Photoperiod timer
	Dissolved oxygen meter
	pH meter
	Ion-specific meter
	Ammonia electrode (or ammonia test kit)
	Specific-conductance meter
	Drying oven
	Desiccator
	Balance (0.01-mg sensitivity)
	Blender
	Refrigerator
	Freezer
	Light box
	Hemacytometer (HA)
	Paper shredder, cutter, or scissors (CT)
	Temperature controller
	Thermometer
	Continuous-recording thermometer
	Photoperiod timer
	Electric drill with stainless steel auger (diameter 7.6 cm, overall length 38 cm, auger bit length 25.4 cm (see 10.3)
	See **(1, 128, 129)** for a listing of equipment needed for water-delivery systems
D. Miscellaneous	Ventilation system for test chambers
	Air supply and airstones (oil free and regulated)
	Cotton surgical gauze or cheese cloth (HA)
	Stainless steel screen (No. 60 mesh, for test chambers)
	Glass hole-cutting bits
	Glass glue
	Plastic mesh (110-µm mesh opening; nylon screen[13]110; HA)
	Aluminum weighing pans
	Fluorescent light bulbs
	Nalgene bottles (500 mL and 1000 mL for food preparation and storage)
	Deionized water
	Airline tubing
	White plastic dishpan
	Coiled-web material[14]
	Brown or white paper toweling (for substrate; CT)
	Screening material (for example, nylon screen[13](110 mesh), window screen, or panty hose; CT)
	Water squirt bottle
	Shallow pans (plastic (light-colored), glass, stainless steel)
E. Chemicals	Detergent (nonphosphate)
	Acetone (reagent grade)
	Hexane (reagent grade)
	Hydrochloric acid (reagent grade)
	Copper Sulfate
	Potassium Chloride
	Reagents for reconstituting water
	Formalin (or other substitutes for formalin, see13.3.7.1)
	Sucrose

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for some experimental designs. Sampling may cause loss of sediment integrity, change in chemical speciation, or disruption of chemical equilibrium (Guide E 1391). A benthic grab or core should be used rather than a dredge to minimize disruption of the sediment sample. Sediment should be collected from a depth that will represent expected exposure. For example, oligochaetes may burrow 4 to 15 cm into sediment.

10.1.3 Exposure to direct sunlight during collection should be minimized, especially if the sediment contains photolytic compounds (**92, 93**). Sediment samples should be cooled to 4°C in the field before shipment (Guide E 1367). Dry ice can be used to cool samples in the field; however, sediments should never be frozen. Monitors can be used to measure temperature during shipping (**1**).

10.1.4 For additional information on sediment collection and shipment see Guide E 1391, USEPA (**61**), and USEPA (**131**) for additional guidance.

10.2 *Storage*:

10.2.1 Since the chemicals of concern and influencing sediment characteristics are not always known, it is desirable to hold the sediments after collection in the dark at 4°C. Traditional convention has held that toxicity tests should be started as soon as possible following collection from the field, although actual recommended storage times range from two weeks (Guide E 1391) to less than eight weeks (USEPA-USACE) (**86**). Discrepancies in recommended storage times reflected a lack of data concerning the effects of long-term storage on the physical, chemical, and toxicological characteristics of the sediment. However, numerous studies have recently been conducted to address issues related to sediment storage (Dillon et al. (**138**); Becker et al. (**406**), Carr and Chapman (**132**), Moore et al. (**133**), Sarda and Burton (**134**), Sijm et al. (**135**), DeFoe and Ankley (**136**)). The conclusions and recommendations offered by these studies vary substantially and appear to depend primarily upon the type or class of chemical(s) present. Considered collectively, these studies suggest that the recommended guidance that sediments be tested sometime between the time of collection and 8 weeks storage is appropriate. Additional guidance is provided below.

10.2.2 Extended storage of sediments that contain high concentrations of labile chemicals (for example, ammonia, volatile organics) may lead to a loss of these chemicals and a corresponding reduction in toxicity. Under these circumstances, the sediment should be tested as soon as possible after collection, but not later than within two weeks (Sarda and Burton (**134**)). Sediments that exhibit low-level to moderate toxicity can exhibit considerable temporal variability in toxicity, although the direction of change is often unpredictable (Carr and Chapman (**132**); Moore et al. (**133**); DeFoe and Ankley (**136**)). For these types of sediments, the recommended storage time of <8 weeks may be most appropriate. In some situations, a minimum storage period for low-to-moderately contaminated sediments may help reduce variability. For example, DeFoe and Ankley (**136**) observed high variability in survival during early testing periods (e.g., <2 weeks) in sediments with low toxicity. De Foe and Ankley (**136**) hypothesized that this variability partially reflected the presence of indigenous predators that remained alive during this relatively short storage period. Thus, if predatory species are known to exist, and the sediment does not contain labile contaminants, it may be desirable to store the sediment for a short period before testing (e.g., 2 weeks) to reduce potential for interferences from indigenous organisms. Sediments that contain comparatively stable compounds (e.g., high molecular weight compounds such as PCBs) or which exhibit a moderate-to-high level of toxicity, typically do not vary appreciably in toxicity in relation to storage duration (Moore et al. (**133**), DeFoe and Ankley (**136**)). For these sediments, long-term storage (e.g., >8 weeks) can be undertaken.

10.2.3 Researchers may wish to conduct additional characterizations of sediment to evaluate possible effects of storage. Concentrations of chemicals of concern could be measured periodically in pore water during the storage period and at the start of the sediment test (**17**). Ingersoll et al. (**14**) recommend conducting a toxicity test with pore water within two weeks from sediment collection and at the start of the sediment test. Freezing might further change sediment properties such as grain size or chemical partitioning and should be avoided (Guide E 1391; Schuytema et al. (**140**)). Sediment should be stored with no air over the sealed samples (no head space) at 4°C before the start of a test (Shuba et al. (**141**)). Sediment may be stored in containers constructed of suitable materials as outlined in Section 9.

10.3 *Manipulation*:

10.3.1 *Homogenization*:

10.3.1.1 Samples tend to settle during shipment. As a result, water above the sediment should not be discarded, but should be mixed back into the sediment during homogenization. Sediment samples should not be sieved to remove indigenous organisms unless there is a good reason to believe they will influence the response of the test organisms. Large indigenous organisms and large debris can be removed using forceps. Reynoldson et al (**95**) observed reduced growth of amphipods, midges, and mayflies in sediments with elevated numbers of oligochaetes and recommended sieving sediments suspected to have high numbers of indigenous oligochaetes. If sediments must be sieved, it may be desirable to analyze samples before and after sieving (for example, pore-water metals, dissolved organic carbon (DOC), acid volatile sulfide (AVS), total organic carbon (TOC)) to document the influence of sieving on sediment chemistry.

10.3.1.2 If sediment is collected from multiple field samples, the sediment can be pooled and mixed using stirring or a rolling mill, feed mixer, or other suitable apparatus (see Guide E 1391). Homogenization of sediment can be accomplished using a hand-held drill outfitted with a stainless steel auger (diameter 7.6 cm, overall length 38 cm, auger bit length 25.4 cm (**17**)).

10.3.2 *Sediment Spiking*:

10.3.2.1 Test sediment can be prepared by manipulating the properties of a control sediment. Mixing time (**63**) and aging (**35, 71, 72**) of spiked sediment can affect bioavailability of chemicals in sediment. Many studies with spiked sediment are often started only a few days after the chemical has been added to the sediment. This short time period may not be long enough for sediments to equilibrate with the spiked chemical. Section

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10.3.2.3). Consistent spiking procedures should be followed in order to make interlaboratory comparisons. Limited studies have been conducted comparing appropriate methods for spiking chemicals in sediment. Additional research is needed before more definitive recommendations for spiking of sediment can be outlined in this standard. The guidance provided in the following sections has been developed from a variety of sources. Spiking procedures that have been developed using one sediment or test organism may not be applicable to other sediments or test organisms. See USEPA (**61**) and Guide E 1391 for additional detail regarding sediment spiking techniques.

(*1*) The cause of sediment toxicity and the interactive effects of chemicals can be determined by spiking a sediment with chemicals or complex waste mixtures (**97**). Sediments spiked with a range of concentrations can be used to generate either point estimates (for example, LC50) or a minimum concentration at which effects are observed (lowest-observable-effect concentration; LOEC). Results of tests may be reported in terms of a BSAF (Biota-sediment accumulation factor; (**142**). The influence of sediment physico-chemical characteristics on chemical toxicity can also be determined with sediment-spiking studies (**47**).

10.3.2.2 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 started, the following should be known about the test material: (*1*) the identity and concentration of major ingredients and impurities, (*2*) water solubility in test water, (*3*) log Kow, BCF (from other test species), persistence, hydrolysis, and photolysis rates of the test substrate, (*4*) estimated toxicity to the test organism and to humans, (*5*) if the test concentration(s) are to be measured, the precision and bias of the analytical method at the planned concentration(s) of the test material, and (*6*) recommended handling and disposal procedures. Addition of test material(s) to sediment may be accomplished using various methods, such as a: (1) rolling mill, (2) feed mixer, or (3) hand mixing (Guide E 1391; USEPA(**61**)). Modifications of the mixing techniques might be necessary to allow time for a test material to equilibrate with the sediment. Mixing time of spiked sediment should be limited from minutes to a few hours and temperature should be kept low to minimize potential changes in the physico-chemical and microbial characteristics of the sediment (Guide E 1391, (**131**)). Duration of contact between the chemical and sediment can affect partitioning and bioavailability (**71**). Care should be taken to ensure that the chemical is thoroughly and evenly distributed in the sediment. Analyses of sediment subsamples is advisable to determine the degree of mixing homogeneity (**143**). Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments (**144**).

10.3.2.2.1 Organic chemicals have been added: (1) directly in a dry (crystalline) form; (2) coated on the inside walls of the container (Ditsworth et al. (**143**)); or (3) coated onto silica sand (e.g., 5 % w/w of sediment) which is added to the sediment (D.R. Mount, USEPA, Duluth, MN, personal communication). In techniques 2 and 3, the chemical is dissolved in solvent, placed in a glass spiking container (with or without sand), then the solvent is slowly evaporated. The advantage of these three approaches is that no solvent is introduced to the sediment, only the chemical being spiked. When testing spiked sediments, procedural blanks (sediments that have been handled in the same way, including solvent addition and evaporation, but contain no added chemical) should be tested in addition to regular negative controls.

10.3.2.2.2 Metals are generally added in an aqueous solution (Guide E 1391; Di Toro et al. (**38**)). Ammonia has also been successfully spiked using aqueous solutions (Besser et al. (**28**)). Inclusion of spiking blanks is recommended.

10.3.2.2.3 Sufficient time should be allowed after spiking for the spiked chemical to equilibrate with sediment components. For organic chemicals, it is recommended that the sediment be aged at least one month before starting a test. Two months or more may be necessary for chemicals with a high log Kow (e.g., >6; D.R. Mount, USEPA, Duluth, MN, personal communication). For metals, shorter aging times (1 to 2 weeks) may be sufficient. Periodic monitoring of chemical concentrations in pore water during sediment aging is highly recommended as a means to assess the equilibration of the spiked sediments. Monitoring of pore water during spiked sediment testing is also recommended.

10.3.2.3 Organic solvents such as triethylene glycol, methanol, ethanol, or acetone may be used, but they might affect TOC levels, introduce toxicity, alter the geochemical properties of the sediment, or stimulate undesirable growths of microorganisms (Guide E 1391). Acetone is highly volatile and might leave the system more readily than triethylene glycol, methanol, or ethanol. 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.

10.3.2.4 If the test contains both a negative control and a solvent control, the survival, growth, or reproduction of the organisms tested should be compared in the two controls. If a statistically significant difference is detected between the two controls, only the solvent control may be used for meeting the acceptability of the test and as the basis for calculation of results. The negative control might provide additional information on the general health of the organisms tested. If no statistically significant difference is detected, the data from both controls should be used for meeting the acceptability of the test and as the basis for calculation of results (Guide E 1241). If performance in the solvent control is markedly different from that in the negative control, it is possible that the data are compromised by experimental artifacts and may not accurately reflect the toxicity of the chemical in natural sediments.

10.3.3 *Test Concentration(s) for Laboratory-spiked Sediments*:

(1) If a test is intended to generate an LC50, a toxicant concentration series (0.5 or higher) should be selected that will provide partial mortality at two or more concentrations of the test chemical. The LC50 of a particular compound may vary depending on physical and chemical sediment characteristics. It may be desirable to conduct a range-finding test in which the

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organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of ten. Results from water-only tests could be used to establish concentrations to be tested in a whole-sediment test based on predicted pore-water concentrations (**39**). See Section 15 for a description of procedures to analyze data generated from these studies.

(2) Bulk-sediment chemical concentrations might be normalized to factors other than dry weight. For example, concentrations of nonpolar organic compounds might be normalized to sediment organic-carbon content and simultaneously extracted metals might be normalized to acid-volatile sulfides (**38**, **39**).

(3) In some situations it might be necessary to simply determine whether a specific concentration of test material is toxic to the test organism, or whether adverse effects occur above or below a specific concentration. When there is interest in a particular concentration, it might only be necessary to test that concentration and not to determine an LC50.

10.4 *Characterization*:

10.4.1 All sediments should be characterized for at least: pH and ammonia of the pore water, organic carbon content (total organic carbon, TOC), particle size distribution (percent sand, silt, clay), and percent water content (Guide E 1367, (**145**)). See Section 10.4.4.7 for a description of procedures for isolating interstitial water.

10.4.2 Other analyses on sediments might include: biological oxygen demand, chemical oxygen demand, cation exchange capacity, Eh, total inorganic carbon, total volatile solids, acid volatile sulfides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and interstitial water analyses.

10.4.3 Macrobenthos may be evaluated by subsampling the field-collected sediment. If direct comparisons are to be made, subsamples for toxicity testing should be collected from the same sample for analysis of sediment physical and chemical characterizations. Qualitative descriptions of the sediment may include color, texture, and presence of macrophytes or animals. Monitoring the odor of sediment samples should be avoided because of potential hazardous volatile chemicals.

10.4.4 *Analytical Methodology*:

10.4.4.1 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.

10.4.4.2 The precision, accuracy, and bias of each analytical method used should be determined in the appropriate matrix: that is, sediment, water, and tissue. Reagent blanks and analytical standards should be analyzed and recoveries should be calculated.

10.4.4.3 Concentration of spiked test material(s) in sediment, interstitial water, and overlying water should be measured as often as practical during a test. If possible, the concentration of the test material in overlying water, interstitial water, and sediments should be measured at the start and end of a test. Measurement of test material(s) degradation products might also be desirable.

10.4.4.4 Separate chambers should be set up at the start of a test and destructively sampled during and at the end of the test to monitor sediment chemistry. Test organisms and food should be added to these extra chambers.

10.4.4.5 Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from about 1 to 2 cm above the sediment surface in the test chamber. Overlying water samples should not contain any surface debris, any material from the sides of the test chamber, or any sediment.

10.4.4.6 Measurement of test material(s) concentration in sediment at the end of a test can be taken by siphoning most of the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the sediment for chemical analysis.

10.4.4.7 *Interstitial water*—Interstitial water (pore water), defined as the water occupying the spaces between sediment or soil particles, is often isolated to provide either a matrix for toxicity testing or to provide an indication of the concentration or partitioning of chemicals within the sediment matrix. Draft USEPA sediment equilibrium partitioning guidelines (ESGs) are based on the presumption that the concentration of chemicals in the interstitial water are correlated directly to their bioavailability and, therefore, their toxicity (Di Toro et al. (**39**)). Of additional importance is contaminants in interstitial waters can be transported into overlying waters through diffusion, bioturbation, and resuspension processes (Van Rees et al. (**405**)). The usefulness of interstitial water sampling for determining chemical contamination or toxicity will depend on the study objectives and nature of the sediments at the study site.

10.4.4.7.1 Isolation of sediment interstitial water can be accomplished by a wide variety of methods, which are based on either physical separation or on diffusion/equilibrium. The common physical-isolation procedures can be categorized as: (1) centrifugation, (2) compression/squeezing, or (3) suction/vacuum. Diffusion/equilibrium procedures rely on the movement (diffusion) of pore-water constituents across semipermeable membranes into a collecting chamber until an equilibrium is established. A description of the materials and procedures used in the isolation of pore water is included in the reviews by Bufflap and Allen (**146**), Guide E1391, and USEPA (**61**).

10.4.4.7.2 When relatively large volumes are required (>20 mL) for toxicity testing or chemical analyses, appropriate quantities of sediment are generally collected with grabs or corers for subsequent isolation of the interstitial water. Several isolation procedures, such as centrifugation (Ankley and Scheubauer-Berigan, (**27**)), squeezing (Carr and Chapman, (**132**)) and suction (Winger and Lasier, (**147**); Winger et al., (**147**)), have been used successfully to obtain adequate volumes for testing purposes. Peepers (dialysis) generally do not produce sufficient volumes for most analyses; however, larger sized peepers (500 mL volume) have been used for collecting interstitial water *in situ* for chemical analyses and organism exposures (Burton, (**148**); Sarda and Burton, (**134**)).

10.4.4.7.3 There is no one superior method for the isolation of interstitial water used for toxicity testing and associated chemical analyses. Factors considered in the selection of an

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isolation procedure may include: (1) volume of pore water needed, (2) ease of isolation (materials, preparation time, and time required for isolation), and (3) artifacts in the pore water caused by the isolation procedure. Each approach has unique strengths and limitations (Bufflap and Allen, (**149**), (**146**); Winger et al., (**147**)), which vary with sediment characteristics, chemicals of concern, toxicity test methods, and desired test resolution (i.e., data quality objectives). For suction or compression separation which use a filter or a similar surface, there may be changes to the characteristics of the interstitial water compared to separation using centrifugation (Ankley et al., (**57**); Horowitz et al. (**392**)) . For most toxicity test procedures, relatively large volumes of interstitial water (e.g., liters) are frequently needed for static or renewal exposures with the associated water chemistry analyses. While centrifugation can be used to generate large volumes of interstitial water, it is difficult to use centrifugation to isolate water from coarser sediment. If smaller volumes of interstitial water are adequate and logistics allow, the use of peepers which establish an equilibrium with the pore water through a permeable membrane may be desirable. If logistics do not allow placement of peeper samplers, an alternative procedure could be to collect cores which are can be sampled using side port suctioning or centrifugation (G.A. Burton, Wright State University, personal communication). However, if larger samples of interstitial water are needed, it would be necessary to collect multiple cores as quickly as possible using an inert environment and centrifugation at ambient temperatures. See USEPA (**136**) and Guide E1391 for additional detail regarding isolation of interstitial water.

10.4.4.7.4 There is no one superior method for the isolation of interstitial water for toxicity testing purposes. Each approach has unique strengths and limitations which vary with the characteristics of the sediment, the chemicals of concern, the toxicity test methods to be used, and the resolution necessary (i.e., the data quality objectives). For suction or compression separation which use a filter or a similar surface, there may be changes to the characteristics of the interstitial water compared to separation using centrifugation (Ankley et al. (**57**). For most toxicity test procedures, relatively large volumes of interstitial water (e.g., liters) are frequently needed for static or renewal exposures with the associated water chemistry analyses. While centrifugation can be used to generate large volumes of interstitial water, it is difficult to use centrifugation to isolate water from coarser sediment. If smaller volumes of interstitial water are adequate and logistics allow, the use of peepers which establish an equilibrium with the pore water through a permeable membrane may be desirable. If logistics do not allow placement of peeper samplers, an alternative procedure could be to collect cores which are can be sampled using side port suctioning or centrifugation (G.A. Burton, Wright State University, personal communication). However, if larger samples of interstitial water are needed, it would be necessary to collect multiple cores as quickly as possible using an inert environment and centrifugation at ambient temperatures. See USEPA (**61**) and Guide E 1391 for additional detail regarding isolation of interstitial water.

11. Quality Assurance and Quality Control

11.1 *Introduction*:

11.1.1 Developing and maintaining a laboratory Quality Assurance (QA) program requires an ongoing commitment by laboratory management and also includes the following: (*1*) appointment of a laboratory quality assurance officer with the responsibility and authority to develop and maintain a QA program, (*2*) preparation of a Quality Assurance Project Plan with Data Quality Objectives, (*3*) preparation of written descriptions of laboratory Standard Operating Procedures (SOPs) for test organism culturing, testing, instrument calibration, sample chain-of-custody, laboratory sample tracking system, and (*4*) provision of adequate, qualified technical staff and suitable space and equipment to ensure reliable data (USEPA (**407**)).

11.1.2 Quality Assurance (QA) practices within a testing laboratory should address all activities that affect the quality of the final data, such as: (*1*) sediment sampling and handling, (*2*) the source and condition of the test organisms, (*3*) condition and operation of equipment, (*4*) test conditions, (*5*) instrument calibration, (*6*) replication, (*7*) use of reference toxicants, (*8*) record keeping, and (*9*) data evaluation.

11.1.3 Quality Control (QC) practices, on the other hand, consist of the more focused, routine, day-to-day activities carried out within the scope of the overall QA program. For more detailed discussion of quality assurance, and general guidance on good laboratory practices related to testing, see (**2, 114, 150, 152-159**).

11.2 *Performance-based Criteria*:

11.2.1 The USEPA Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards for chemical analytical methods (**88**). Performance-based methods were defined by EMMC as a monitoring approach which permits the use of appropriate methods that meet preestablished demonstrated performance standards. Minimum required elements of performance, such as precision, reproducibility, bias, sensitivity, and detection limits should be specified and the method should be demonstrated to meet the performance standards.

11.2.2 In developing guidance for culturing test organisms to be included in this standard for sediment tests, it was generally agreed that no single method must be used to culture organisms. Success of a test relies on the health of the culture from which organisms are taken for testing. Having healthy organisms of known quality and age for testing is the key consideration relative to culture methods. Therefore, a performance-based criteria approach is the preferred method through which individual laboratories can evaluate culture health rather than requiring all laboratories to use the same culturing procedure. Performance-based criteria were chosen in USEPA (**1**) and in this standard to allow each laboratory to optimize culture methods while providing organisms that produce reliable and comparable test results. See 13.1.2, 14.1.2, and Annex A1 to Annex A7 for a listing of performance criteria for culturing and testing.

11.3 *Facilities, Equipment, and Test Chambers*:

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11.3.1 Separate test organism culturing and testing areas must be provided to avoid loss of cultures because of cross-contamination. Ventilation systems should be designed and operated to prevent recirculation or leakage of air from chemical analysis laboratories or sample storage and preparation areas into test organism culturing or sediment testing areas, and from sediment testing laboratories and sample preparation areas into culture areas.

11.3.2 Equipment for temperature control should be adequate to maintain recommended test-water temperatures. Recommended materials should be used in the fabrication of the test equipment which comes in contact with the sediment or overlying water.

11.3.3 Before a sediment test is conducted in a new facility, a "non-contaminant" test should be conducted in which all test chambers contain a control sediment and overlying water. This information is used to demonstrate that the facility, control sediment, water, and handling procedures provide acceptable responses of test organisms (see 11.14).

11.4 *Test Organisms*— The organisms should appear healthy, behave normally, feed well, and have low mortality in cultures, during holding (for example, <20 % for 48 h before the start of a test), and in test controls. Test organisms should be positively identified to species. Obtaining wild populations of organisms for testing should be avoided unless the ability of the wild population to cross-breed with existing laboratory populations has been determined (see 12.2.2).

11.5 *Water*—The quality of water used for organism culturing and testing is extremely important. Overlying water used in testing and water used in culturing organisms should be uniform in quality. Acceptable water should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (for example, discoloration, unusual behavior). See Section 7 for additional details.

11.6 *Sample Collection and Storage*—Sample holding times and temperatures should conform to conditions described in Section 10.

11.7 *Test Conditions*— It is desirable to measure temperature continuously in at least one chamber during each test. Temperatures should be maintained within the limits specified for each test. Dissolved oxygen, alkalinity, water hardness, conductivity, ammonia, and pH should be checked in accordance with 13.3, 14.3, or in Annex A1 to Annex A7.

11.8 *Quality of Test Organisms*:

11.8.1 It may be desirable for laboratories to periodically perform 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms (See Table 10 and Table 11 and Section 11.16). Data from these reference-toxicity tests could be used to assess genetic strain or life-stage sensitivity to select chemicals. The previous requirement for laboratories to conduct monthly reference-toxicity tests in an earlier version of this standard (Test Method E 1706-95b) has not been included as a requirement for testing sediments due to the inability of reference-toxicity tests to identify stressed populations of test organisms (McNulty et al. (**98**)). Physiological measurements such as lipid content might also provide useful information regarding the health of the cultures.

TABLE 10 Recommended Test Conditions for Conducting Reference-Toxicity Tests With One Organism/Chamber

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, Cd, or Cu
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:	None
9. Age of organisms:	*H. azteca*: 7- to 14-day old (within a 1- to 2-day range in age) *C. tentans*: second-to third-instar larvae (about 10-day-old larvae)(Section 12.4.1) *L. variegatus:* adults
10. Test chamber:	30-mL plastic cups (covered with glass or plastic)
11. Volume of water:	20 mL
12. Number of organisms/chamber:	1
13. Number of replicate chambers/treatment:	10 minimum
14. Feeding:	*H. azteca*: 0.1 mL YCT (1800 mg/L stock) on Day 0 and 2 *C. tentans*: 0.25 Tetrafin® (4 g/L stock) on Day 0 and 2 *L. variegatus:* not fed
15. Substrate:	*H. azteca*: Nitex® screen (110 mesh) *C. tentans*: sand (monolayer) *L. variegatus:* no substrate
16. Aeration:	None
17. Dilution water:	Culture water, well water, surface water, site water, or reconstituted water
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:	96 h
21. Endpoint:	Survival (LC50)
22. Test acceptability:	90 % control survival

11.8.2 It is desirable to determine the sensitivity of test organisms obtained from an outside source. The supplier should provide data with the shipment describing the history of the sensitivity or organisms from the same source culture. The supplier should also certify the species identification of the test organisms, and provide the taxonomic references, or name(s) of the taxonomic expert(s) consulted.

11.8.3 All organisms in a test must be from the same source (Section 10.2.2). Organisms may be obtained from laboratory cultures or from commercial or government sources. The test organism used should be identified using an appropriate taxonomic key, and verification should be documented (e.g., (**164**), Merritt and Cummins (**394**)). Obtaining organisms from wild populations should be avoided unless organisms are cultured through several generations in the laboratory. In addition, the ability of the wild population of sexually reproducing organisms to cross-breed with the existing laboratory population should be determined (Duan et al., (**163**)). Sensitivity of the wild population to select chemicals (for example, Table 2) should also be documented.

11.9 *Quality of Food*— Problems with the nutritional suitability of the food will be reflected in the survival, growth, or reproduction of the test organisms in cultures or in sediment tests.

11.10 *Test Acceptability*:

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TABLE 11 Recommended Test Conditions for Conducting Reference-Toxicity Tests With More Than One Organism/ Chamber

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, Cd, or Cu
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:	None
9. Age of organisms:	*H. azteca*: 7- to 14-day old (within a 1- to 2-day range in age) *C. tentans*: second- to third-instar (about 10-day old larvae)(Section 12.4.1) *L. variegatus:* adults
10. Test chamber:	250-mL glass beaker (covered with glass or plastic)
11. Volume of water:	100 mL (minimum)
12. Number of organisms/ chamber:	10 minimum
13. Number of replicate chambers/treatment:	3 minimum
14. Feeding:	*H. azteca*: 0.5 mL YCT (1800 mg/L stock) on Day 0 and 2 *C. tentans*: 1.25 mL Tetrafin® (4 g/L stock) on Day 0 and 2 *L. variegatus:* not fed
15. Substrate:	*H. azteca*: Nitex® screen (110 mesh) *C. tentans*: sand (monolayer) *L. variegatus*: no substrate
16. Aeration:	None
17. Dilution water:	Culture water, well water, surface water, site water, or reconstituted water
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:	96 h
21. Endpoint:	Survival (LC50)
22. Test acceptability:	90 % control survival

11.10.1 Section 13.2 and 14.2 and Annex A1 to Annex A7 outline requirements for acceptability of tests. An individual test may be conditionally acceptable if temperature, dissolved oxygen, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of a test will depend on the experience and professional judgment of the laboratory analyst and the reviewing staff of the regulatory authority. Any deviation from test specifications should be noted when reporting data from a test.

11.11 *Analytical Methods*:

11.11.1 All routine chemical and physical analyses for culture and testing water, food, and sediment should include established quality assurance practices (**153-156**).

11.11.2 Reagent containers should be dated when received from the supplier and the shelf life of the reagent should not be exceeded. Working solutions should be dated when prepared and the recommended shelf life should not be exceeded.

11.12 *Calibration and Standardization*:

11.12.1 Instruments used for routine measurements of chemical and physical characteristics such as pH, dissolved oxygen, temperature, and conductivity should be calibrated before use each day according to the instrument manufacturer's procedures as indicated in the general section on quality assurance (see USEPA Methods 150.1, 360.1, 170.1, and 120.1, (**125**)). Calibration data should be recorded in a permanent log.

11.12.2 A known-quality water should be included in the analyses of each batch of water samples (for example, water hardness, alkalinity, conductivity). It is desirable to include certified standards in the analysis of water samples.

11.13 *Replication and Test Sensitivity*—The sensitivity of sediment tests will depend in part on the number of replicates/ treatment, the significance level selected, and the type of statistical analysis. If the variability remains constant, the sensitivity of a test will increase as the number of replicates is increased. The minimum recommended number of replicates varies with the objectives of the test and the statistical method used for analysis of the data (see Section 15).

11.14 *Demonstrating Acceptable Performance*:

11.14.1 Intralaboratory precision, expressed as a coefficient of variation, of the range for each type of test to be used in a laboratory can be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (for example, the same test duration, type of water, age of test organisms, feeding), and same data analysis methods. A reference-toxicant concentration series (0.5 or higher) should be selected that will provide partial mortalities at two or more concentrations of the test chemical (see Section 10.3.3). Information from previous tests can be used to improve the design of subsequent tests to optimize the dilution series selected for testing.

11.14.2 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 (i.e., water quality) result in acceptable performance in the tests as outlined in Sections 13 and 14 and in Annex A1 to Annex A7.

11.14.3 Laboratories should demonstrate that their personnel are able to recover an average of at least 90 % of the organisms from whole sediment. For example, test organisms could be added to control or test sediments, and recovery could be determined after 1 h (**160**).

11.15 *Documenting Ongoing Laboratory Performance*:

11.15.1 Outliers, which are data falling outside the control limits and trends of increasing or decreasing sensitivity are readily identified. If the data from a given test falls outside the "expected" range (for example, ±2 SD), the sensitivity of the organisms and the credibility of the test results may be suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

11.15.2 A sediment test may be acceptable if specified conditions of a reference-toxicity test fall outside the expected ranges (see 11.10). Specifically, a sediment test should not be judged unacceptable if the LC50 for a given reference-toxicity test falls outside the expected range or if control survival in the reference-toxicity test is <90 %. All the performance criteria outlined in 13.1.2 and 14.1.2 or in Annex A1 to Annex A7 must

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be considered when determining the acceptability of a sediment test. The acceptability of the sediment test would depend on the experience and judgment of the investigator and the regulatory authority.

11.15.3 Performance should improve with experience, and the control limits should gradually narrow, as the statistics stabilize. However, control limits of ±2 SD, by definition, will be exceeded 5 % of the time, regardless of how well a laboratory performs. For this reason, good laboratories that develop very narrow control limits may be penalized if a test result which falls just outside the control limits is rejected *de facto*. The width of the control limits should be considered in decisions regarding rejection of data (see Section 17).

11.16 *Reference-toxicity Testing*:

11.16.1 Historically, reference-toxicant testing has been thought to provide three types of information relevant to the interpretation of toxicity test data: (1) an indication of the relative "health" of the organisms used in the test; (2) a demonstration that the laboratory can perform the test procedure in a consistent manner; and (3) information to indicate whether the sensitivity of the particular strain or population in use at a laboratory is comparable to those in use in other facilities. With regard to the first type of information, recent work by McNulty et al. (**98**) suggests that reference-toxicant tests may not be effective in identifying stressed populations of test organisms. In addition, reference-toxicant tests recommended for use with sediment toxicity tests are short-term, water column tests, owing in part to the lack of a standard sediment for reference-toxicity testing. Because the test procedures for reference-toxicant tests are not the same as for the sediment toxicity tests of interest, the applicability of reference-toxicity tests to demonstrate ability to reproducibly perform the sediment test procedures is greatly reduced. Particularly for the long-term sediment toxicity tests with *H. azteca* and *C. tentans*, performance of control organisms over time may be a better indicator of success in handling and testing these organisms (Annex A6 and Annex A7).

11.16.2 Although the requirement for monthly testing has been removed in this current version of this standard, periodic reference testing should still be conducted as an indication of overall comparability of results among laboratories (at a minimum, 6 tests over a 3 year period should be conducted to evaluate potential differences in life stage or genetic strain of test organisms). In particular, reference-toxicity tests should be performed more frequently when organisms are obtained from outside sources, when there are changes in culture practices, or when brood stock from an outside source is incorporated into a laboratory culture.

11.16.3 Reference toxicants such as potassium chloride (KCl), cadmium chloride ($CdCl_2$), and copper sulfate ($CuSO_4$) are suitable for use. (See Table 10 and Table 11.) No one reference toxicant can be used to measure the condition of test organisms in respect to another toxicant with a different mode of action (**161**). However, it may be unrealistic to test more than one or two reference toxicants routinely. Potassium chlorine has been used successfully in round-robin water-only exposures with *H. azteca* and *C. tentans* (see Section 17).

11.16.4 Test conditions for conducting reference-toxicity tests with *H. azteca* and *C. tentans* are outlined in Tables 10 and 11. The procedures outlined in Tables 10 and 11 can also be used for conducting reference-toxicity tests with the test organisms outlined in Annex A1 to Annex A7. Reference-toxicity tests can be conducted using one organism/chamber or multiple organisms in each chamber. Some laboratories have observed low control survival when more than one midge/chamber is tested in water-only exposures.

11.17 *Record Keeping*— Section 15.1 outlines recommendations for recorded keeping (i.e., data files, chain-of custody).

12. Collection, Culturing, and Maintaining Test Organisms

12.1 *Life Histories*— The following sections describe life histories and culturing procedures for *Hyalella azteca* and *Chironomus tentans*. Annex A1 to Annex A7 describe life histories and culturing procedures for the additional test organisms.

12.1.1 *Hyalella azteca*:

12.1.1.1 *Hyalella azteca* inhabit permanent lakes, ponds, and streams throughout North and South America (**162, 164**). Occurrence of *H. azteca* is most common in warm (20 to 30°C for much of the summer) mesotrophic or eutrophic lakes which support aquatic plants. This amphipod is also found in ponds, sloughs, marshes, rivers, ditches, streams, and springs, but in lower numbers. *Hyalella azteca* have achieved densities of >10 000 m^{-2} in preferred habitats (**162**).

12.1.1.2 *Hyalella azteca* is an epibenthic detritivore that burrows into the sediment surface. Hargrave (**165**) reported that *H. azteca* selectively ingests bacteria and algae. The behavior and feeding habits of *H. azteca* make them excellent test organism for sediment assessments.

12.1.1.3 Reproduction by *H. azteca* is sexual. The adult males are larger than females and males have larger second gnathopods (**162**). Males pair with females by grasping the females (amplexus) with their gnathopods while on the backs of the females. After feeding together for 1 to 7 days the female is ready to molt and the two organisms separate for a short time while the female sheds her old exoskeleton. Once the exoskeleton is shed, the two organisms reunite and copulation occurs. The male places sperm near the marsupium of the female and her pleopods sweep the sperm into the marsupium. The organisms again separate and the female releases eggs from her oviducts into the marsupium where the eggs are fertilized. *Hyalella azteca* average about 18 eggs/brood (**164**) with larger organisms having more eggs (**166**).

12.1.1.4 The developing embryos and newly hatched young are kept in the marsupium until the next molt. At 24 to 28°C, hatching ranges from 5 to 10 days after fertilization (**166-168**). The time between molts for females is 7 to 8 days at 26 to 28°C (**168**). Therefore, about the time embryos hatch, the female molts and releases the young. *Hyalella azteca* average 15 broods in 152 days (**164**). Pairing of the sexes is simultaneous with embryo incubation of the previous brood in the marsupium. *Hyalella azteca* have a minimum of nine instars (**169**). There are 5 to 8 pre-reproductive instars (**166**) and an indefinite number of post-reproductive instars. The first five instars form the juvenile stage of development, instar stages 6 and 7 form

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the adolescent stage when sexes can be differentiated, instar Stage 8 is the nuptial stage and all later instars are the adult stages of development (**164**).

12.1.1.5 *Hyalella azteca* have been successfully cultured at illuminance of about 100 to 1000 l× (**10**, **102**, **170**). *Hyalella azteca* avoid bright light, preferring to hide under litter during the day and feed.

12.1.1.6 Temperatures tolerated by *H. azteca* range from 0 to 33°C (**167**, **168**, **171**). At temperatures <10°C the organisms rest and are immobile (**172**, **173**). At temperatures of 10 to 18°C, reproduction can occur. Juveniles grow more slowly at colder temperatures and become larger adults. Smaller adults with higher reproduction are typical when organisms are grown at 18 to 28°C. The highest rates of reproduction occur at 26 to 28°C (**173**) while lethality occurs at 33 to 37°C (**171**, **174**).

12.1.1.7 *Hyalella azteca* are found in waters of widely varying types. *Hyalella azteca* can inhabit saline waters up to 29 ppt; however, their distribution in these saline waters has been correlated to water hardness (**115**). *Hyalella azteca* inhabit water with high magnesium concentrations at conductivities up to 22 000 µS/cm, but only up to 12 000 µS/cm in sodium-dominated waters (**115**). de March (**162**) reported *H. azteca* were not collected from locations where calcium was <7 mg/L. *Hyalella azteca* have been cultured in water with a salinity up to 15 ppt in reconstituted salt water (**115**, **118**). In laboratory studies, Sprague (**171**) reported a 24-h LC50 for dissolved oxygen at 20°C of 0.7 mg/L. Pennak and Rosine (**175**) reported similar findings. Nebeker et al (**176**) reported 48-h and 30-day LC50s for *H. azteca* of <0.3 mg/L dissolved oxygen. Weight and reproduction of *H. azteca* were reduced after 30-day exposure to 1.2 mg/L dissolved oxygen.

12.1.1.8 *Hyalella azteca* tolerate a wide range of substrates. Ingersoll and Nelson (**102**) and Ingersoll et al (**81**) reported that *H. azteca* tolerated sediments ranging from more than 90 % silt- and clay-sized particles to 100 % sand-sized particles without detrimental effects on either survival or growth. *Hyalella azteca* tolerated a wide range in grain size and organic matter in 10- to 42-day tests with formulated sediment (**59**; Ingersoll et al., (**55**)). Ankley et al (**57**) evaluated the effects of natural sediment physico-chemical characteristics on the results of 10-day laboratory toxicity tests with *H. azteca* and *C. tentans*. Tests were conducted with and without the addition of exogenous food. Survival of organisms was decreased in tests without added food. Physico-chemical sediment characteristics including grain size and TOC were not significantly correlated to the response of *H. azteca* in either fed or unfed tests. See Section A6.4 for additional detail regarding studies of the influence grain size in long-term sediment toxicity tests with *H. azteca*.

12.1.2 *Chironomus tentans*:

12.1.2.1 *Chironomus tentans* have a holarctic distribution (**177**) and are commonly found in eutrophic ponds and lakes (**178**, **179**). Midge larvae are important in the diet of fish and waterfowl (**180-183**). Larvae of *C. tentans* usually penetrate a few centimeters into sediment. In both lotic and lentic habitats with soft bottoms, about 95 % of the chironomid larvae occur in the upper 10 cm of substrate, very few larvae are found below 40 cm (**177**). Larvae were found under the following conditions in British Columbia lakes by Topping (**184**): particle size <0.15 mm to 2.0 mm, temperature 0 to 23.3°C, dissolved oxygen 0.22 to 8.23 mg/L, pH 8.0 to 9.2, conductivity 481 to 4136 µΩ/cm, and sediment organic carbon 1.9 to 15.5 %. Larvae were absent from lakes if hydrogen sulfide concentration in overlying water exceeded 0.3 mg/L. Abundance of larvae was positively correlated with conductivity, pH, amount of food, percentages of particles in the 0.59 to 1.98-mm size range, and concentrations of Na, K, Mg, Cl, SO_4, and dissolved oxygen. Others (for example, (**185**, **186**)) have reported a temperature range from 0 to 35°C and a pH range from 7 to 10.

12.1.2.2 *Chironomus tentans* are aquatic during the larval and pupal stages. The life cycle of *C. tentans* can be divided into four distinct stages: (*1*) an egg stage, (*2*) a larval stage, consisting of four instars, (*3*) a pupal stage, and (*4*) an adult stage. Mating behavior has been described by Sadler (**180**) others (**1**). Males are easily distinguished from females because males have large, plumose antennae and a much thinner abdomen with visible genitalia. The male has paired genital claspers on the posterior tip of the abdomen (**177**). The adult female weighs about twice as much as the male, with about 30 % of the female weight contributed by eggs. After mating, adult females oviposit a single transparent, gelatinous egg case directly into the water. An egg case contains about 2300 eggs (**180**). At USEPA Duluth, the females oviposit eggs withing 24 h of emergence. Egg cases contain a variable number of eggs from about 500 to 2000 eggs/egg case (USEPA (**1**)) and will hatch in 2 to 4 days at 23°C. Under optimal conditions larvae will pupate and emerge as adults after about 21 days at 23°C. Larvae begin to construct tubes (or cases) on the second or third day after hatching. The cases lengthen and enlarge as the larvae grow with the addition of small particles bound together with threads from the mouths of larvae (**180**). The larvae draw food particles inside the tubes and also feed in the immediate vicinity of either end of the open-ended tubes with their caudal extremities anchored within the tube. The four larval stages are followed by a black-colored pupal stage (lasting about 3 days) and emergence to a terrestrial adult (imago) stage. The adult stage lasts for 3 to 5 days, during which the adults mate during flight and the females oviposit their egg cases (2 to 3 days postemergence, (**180**)).

12.1.2.3 *Chironomus tentans* tolerate a wide range of substrates. Grain size tolerance of *C. tentans* in sediment exposures are described in Section 14.4 for 10-day tests and in A7.4 for long-term exposures.

12.2 *General Culturing Procedures*:

12.2.1 Acceptability of a culturing procedure is based in part on performance of organisms in culture and in the sediment test (see Section 5 and 11.2). No single technique for culturing test organisms is recommended. What may work well for one laboratory may not work as well for another laboratory. While a variety of culturing procedures are outlined in 12.3 for *H. azteca* and in 12.4 for *C. tentans,* organisms must meet the test acceptability requirements listed in 13.1.2 or 14.1.2. Culturing procedures are outlined for the additional test organisms in Annex A1 to Annex A7.

12.2.2 All organisms in a test must be from the same source. Organisms may be obtained from laboratory cultures or from

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commercial or government sources (see Table 12). The test organism used should be identified using an appropriate taxonomic key and verification should be documented. Obtaining organisms from wild populations should be avoided unless organisms are cultured through several generations in the laboratory. In addition, the ability of the wild population to crossbreed with existing laboratory populations should be determined. The sensitivity of the wild population to select chemicals (for example, Table 2) should also be documented (Duan et al. (**163**)).

12.2.3 Test organisms obtained from commercial sources should be shipped in well-oxygenated water in insulated containers to maintain temperature during shipment. Temperature and dissolved oxygen of the water in the shipping containers should be measured on arrival to determine if the organisms might have been subjected to low dissolved oxygen or temperature fluctuations. The temperature of the shipped water should be gradually adjusted to the desired culture temperature at a rate not exceeding 2°C/24 h. Additional reference-toxicity testing is suggested if organisms are not cultured at the testing laboratory (see 11.16).

12.2.4 A group of organisms should not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed (for example, >20 % mortality for 48 h before the start of a test). If the organisms fail to meet these criteria, the entire batch should be discarded and a new batch should be obtained. All organisms should be as uniform as possible in age and life stage. Test organisms should be handled as little as possible.

TABLE 12 Sources of Starter Cultures of Test Organisms

Source	Species
U.S. Environmental Protection Agency Midcontinent Ecological Division 6201 Congdon Boulevard Duluth, MN 55804 Teresa Norberg-King (218/529-5163, fax -5003) email: norberg-king.teresa@epa.gov	*H. azteca* *C. tentans*
U.S. Environmental Protection Agency Environmental Monitoring System Laboratory 26 W. Martin Luther King Dr. Cincinnati, OH 45244 Jim Lazorchak (513/569-7076, fax -7609) email: lazorchak.jim@epa.gov	*H. azteca* *L. variegatus*
Columbia Environmental Research Center 4200 New Haven Road U.S. Geological Survey 4200 New Haven Road Columbia, MO 65201 Eugene Greer (573/876-1820, fax -1896) email: eugene_greer@usgs.gov	*H. azteca* *C. tentans* *L. variegatus*
Wright State University Institute for Environmental Quality Dayton, OH 45435 Allen Burton (513/775-2201, fax -4997) email: aburton@desire.wright.edu	*H. azteca* *C. tentans*
Michigan State University Department of Fisheries and Wildlife No. 13 Natural Resources Building East Lansing, MI 48824-1222 John Giesy (517/353-2000, fax 517/432-1984) email: igiesy@aol.com	*H. azteca* *C. tentans*

When handling is necessary, it should be done as gently, carefully, and as quickly as possible.

12.2.5 *Hyalella azteca, C. tentans*, and the test organisms described in Annex A1 to Annex A7 can be cultured in a variety of waters. Water of a quality sufficient to culture fathead minnows (*Pimephales promelas*) or cladocerans will generally be adequate.

12.2.5.1 Variable success has been reported using reconstituted waters to culture or test *H. azteca* (See 7.1.3).

12.2.5.2 Organisms can be cultured using either static or renewal procedures. Renewal of water is recommended to limit loss of the culture organisms from a drop in dissolved oxygen or a buildup of waste products. In renewal systems, there should be at least one volume addition/day of culture water to each chamber. In static systems, the overlying water volume should be changed at least weekly by siphoning down to a level just above the substrate and slowly adding fresh water. Extra care should be taken to ensure that proper water quality is maintained in static systems. For example, aeration is needed in static systems to maintain dissolved oxygen at >2.5 mg/L.

12.2.5.3 A recirculating system using an under-gravel filter has been used to culture amphipods and midges (**1**). The approach for using a recirculating system to culture organisms has been described by (**188-190**). Under-gravel filters can be purchased from aquarium suppliers and consist of an elevated plate with holes that fit on the bottom of an aquarium. The plate has a standpipe to which a pump can be attached. Gravel or an artificial substrate (for example, plastic balls or multi-plate substrates) are placed on the plate. The substrates provide surface area for microorganisms that use nitrogenous compounds. A simple example of a recirculating system is two aquaria positioned one above the other with a total volume of 120 L. The bottom aquarium contains the under-gravel filter system, gravel, or artificial substrate, and a submersible pump. The top aquarium is used for culture of animals and has a hole in the bottom with a standpipe for returning overflow water to the bottom aquarium. Water lost to evaporation is replaced weekly and water is replaced at one to two-month intervals. Cultures fed foods such as fish food flakes[12] should include limestone gravel to help avoid depression in pH. Recirculating systems require less maintenance than static systems.

12.2.6 Cultures should be maintained at 23°C with a 16L:8D photoperiod at a illuminance of about 100 to 1000 l× (**1**). Cultures should be observed daily. Water temperature should be measured daily or continuously and dissolved oxygen should be measured weekly. It may be desirable for laboratories to periodically perform 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms (see Section 11.16.2). Data from these reference-toxicity tests could be used to assess genetic strain or life-stage sensitivity to select chemicals. The previous requirement for laboratories to conduct monthly reference-toxicity tests in an earlier version of this standard (Test Method E 1706-95b) has not been included as a requirement for testing sediments due to the inability of reference-toxicity tests to identify stressed populations of test organisms (**98**). Culture water hardness, alkalinity, ammonia, and pH should be measured at least quarterly and the day before the start of a sediment test. If reconstituted water is used

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to culture organisms, water quality should be measured on each batch of reconstituted water. Culture procedures should be evaluated and adjusted as appropriate to restore or maintain the health of the culture.

12.3 *Culturing Procedures for Hyalella azteca*:

12.3.1 The culturing procedures described as follows are based on methods described in **(1)**, **(6)**, **(55)**, **(57)**, **(102)**, **(191)**, **(192)**. If the objective of the study is to follow the test method for *H. azteca* outlined in 13.1.2, the culturing procedure must produce 7- to 14-day old amphipods to start the 10-day test (see 13.1.2). The 10-day test with *H. azteca* should start with a narrower range in size or age of *H. azteca* (i.e., 1- to 2-day range in age) to reduce potential variability in growth at the end of a 10-day test. This narrower range would be easiest to obtain using known-age organisms (i.e., section 12.3.2 or 12.3.4) instead of sieving the cultures (Section 12.3.5) to obtain similar-sized amphipods (i.e., amphipods within a range from 1 to 2-days old will be more uniform in size than organisms within a range of 7-days old). The culturing procedure must produce 7- to 8-day-old amphipods to start a long-term test with *H. azteca* (Annex A6).

12.3.2 The following procedure described by Brooke et al **(6)** and USEPA Duluth **(191)** can be used to obtaining known-age amphipods to start a test. Mature amphipods (50 organisms ≥30-days old at 23°C) are held in 2-L glass beakers containing 1 L of aerated culture water. Amphipods are fed 10 mL of the yeast-cereal leaves[10]-trout chow (YCT) mixture (Annex A8) and 10 mL of the green algae *Selenastrum capricornutum* (about 3.5×10^7 cells/mL) on Monday. Five milliliters of food are added to cultures on Wednesdays and Fridays.

12.3.2.1 Water in the culture chambers is changed weekly. Survival of adults and juveniles and production of young amphipods should be measured at this time. The contents of the culture chambers are poured into a translucent white plastic pan or white enamel pan. After the adults are removed, the remaining amphipods (about 200) will range in ages from <1 to 7-days old. Young amphipods are transferred with a pipet into a 1-L beaker containing culture water and are held for one week before starting a toxicity test. Cotton gauze should be soaked in water for 24 h before use and should be renewed weekly. Presoaked cotton gauze is placed in the beakers and organisms are fed 10 mL of YCT and 5 mL of each food each following day (Annex A8). Survival of young amphipods should be >80 % during this one-week holding period. Records should be kept on the number of surviving adults, number of breeding pairs, and young production and survival. This information can be used to develop control charts which are useful in determining if cultures are maintaining a vigorous reproductive rate indicative of culture health. Some of the adult amphipods can be expected to die in the culture chambers, but mortality greater than about 50 % should be cause for concern. Typical reproductive rates in culture chambers containing 60 adults can be as high as 900 young/week. A decrease in reproductive rate may be caused by a change in water quality, temperature, food quality, or brood stock health. Adult females will continue to reproduce for several months.

12.3.3 A second procedure for obtaining known-age amphipods is described by Borgmann et al **(193)**. Known-age amphipods are cultured in 2.5-L chambers containing about 1 L of culture water and between 5 and 25 adult *H. azteca*. Each chamber contains pieces of cotton gauze presoaked in culture water. Once a week the test organisms are isolated from the gauze and collected using a sieve. Amphipods are then rinsed into petri dishes where the young and adults are sorted. The adults are returned to the culture chambers containing fresh water and food.

12.3.4 A third procedure for obtaining known-age amphipods is described by Greer **(192)** and Tomasovic et al **(160, 55)**. Mass cultures of mixed-age amphipods are maintained in 80-L glass aquaria containing about 50 L of water **(102)**. A flaked food (e.g., fish food flakes[12]) are added to each culture chamber receiving daily water renewals to provide about 20 g dry solids/50 L of water twice weekly in a 80-L culture chamber. Additional flaked food is added when most of the flaked food has been consumed. Laboratories using static systems should develop lower feeding rates specific to their systems. Each culture chamber has a substrate of maple leaves and artificial substrates (six 20-cm diameter sections/80-L aquaria of "coiled-web material"[13]). Before use, leaves are soaked in 30 ppt salt water for about 30 days to reduce the occurrence of planaria, snails, or other organisms in the substrate. The leaves are then flushed with water for 1 h to remove the salt water and residuals of naturally occurring tannic acid before placement in the cultures.

12.3.4.1 To obtain known-age amphipods, a U.S. Standard Sieve No. 25 (710-µm mesh) is placed under water in a chamber containing mixed-age amphipods. A No. 25 sieve will retain mature amphipods and immature amphipods will pass through the mesh. Two or three pieces of artificial substrate[13] or a mass of leaves with the associated mixed-age amphipods are quickly placed into the sieve. The sieve is brought to the top of the water in the culture chamber keeping all but about 1 cm of the sieve under water. The artificial substrates or leaves are then shaken under water several times to dislodge the attached amphipods. The artificial substrates or leaves are taken out of the sieve and placed back in the culture chamber. The sieve is agitated in the water to rinse the smaller amphipods back into the culture chamber. The larger amphipods remaining in the sieve are transferred with a pipet into a dish and then placed into a shallow glass pan (for example, a pie pan) where immature amphipods are removed. The remaining mature amphipods are transferred using a pipet into a second No. 25 sieve which is held in a glass pan containing culture water.

12.3.4.2 The mature amphipods are left in the sieve in the pan overnight to collect any newborn amphipods that are released. After 24 h, the sieve is moved up and down several times to rinse the newborn amphipods (<24-h old) into the surrounding water in the pan. The sieve is removed from the pan and the mature amphipods are placed back into their culture chamber or placed in a second glass pan containing culture water if additional organisms are needed for testing.

[13] Nylon screen, such as Nitex, is available from a variety of commercial sources, has been found suitable for this purpose.

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The newborn amphipods are moved with a pipet and placed in a culture chamber with flowing water during a grow-out period. The newborn amphipods should be counted to determine if adequate numbers have been collected for the test. Methods have recently been developed to hold the newborn amphipods using flow-through conditions before the start of a test.

12.3.4.3 Isolation of about 1500 (750 pairs) of adults in amplexus provided about 800 newborn amphipods in 24 h and required about six man-hours of time. Isolation of about 4000 mixed-age adults (some in amplexus and others not in amplexus) provided about 800 newborn amphipods in 24 h and required less than one man-hour of time. The newborn amphipods can be held for 6 to 13 days to provide 7- to 14-day old organisms to start a 10-day test (see Section 13) or should be held for 7 days to provide 7- to 8-day-old organisms to start a long-term test (Annex A6). The neonates are held in a 2-L beaker for 6 to 13 days before the start of a test. On the first day of isolation, the neonates are fed 10 mL of YCT (1800 mg/L stock solution) and 10 mL of *Selanastrum capricornutum* (about 3.5×10^7 cells/mL). On the third, fifth, seventh, ninth, eleventh, and thirteenth days after isolation, the amphipods are fed 5 mL of both YCT and *S. capricornutum*. Amphipods are initially fed a higher volume to establish a layer of food on the bottom of the culture chamber. If dissolved oxygen drops below 4 mg/L, about 50 % of the water should be replaced (Ingersoll et al., (**55**)).

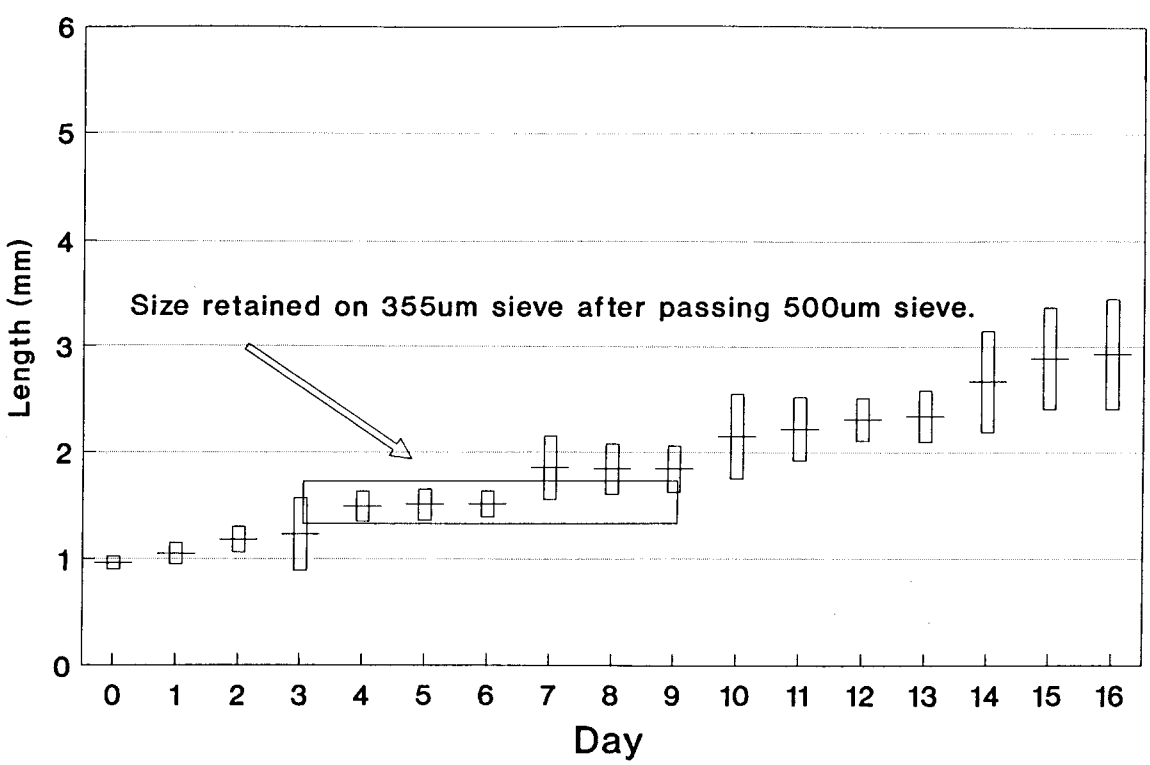
12.3.5 Laboratories that use mixed-age amphipods for testing must demonstrate that the procedure used to isolate amphipods will produce test organisms that are 7 to 14-days old if procedures outlined in 13.1.4 are to be followed. For example, Winger and Lasier (**118**) reported amphipods passing through a U.S. Standard No. 35 sieve (500 µm), but stopped by a No. 45 sieve (355 µm) averaged 1.54 mm (standard deviation (SD) 0.09) in length. The mean length of these sieved organisms corresponds to that of 6-day old amphipods (see Fig. 1). After holding for 3 days before testing to eliminate organisms injured during sieving, these amphipods were about 9-days old (length 1.84 mm, SD 0.11) at the start of a toxicity test.

12.3.5.1 Ingersoll and Nelson (**102**) describe the following procedure for obtaining mixed-age amphipods of a similar size to start a test. Smaller amphipods are isolated from larger amphipods using a stack of U.S. Standard sieves: No. 30 (600 µm), No. 40 (425 µm), and No. 60 (250 µm). Sieves should be held under water to isolate the amphipods. Amphipods may float on the surface of the water if they are exposed to air. Artificial substrate or leaves are placed in the No. 30 sieve. Culture water is rinsed through the sieves and small amphipods stopped by the No. 60 sieve are washed into a collecting pan. Larger amphipods in the No. 30 and No. 40 sieves are returned to the culture chamber. The smaller amphipods are then placed in 1-L beakers containing culture water and food (about 200 amphipods/beaker) with gentle aeration.

12.3.5.2 Amphipods should be held and fed at a similar rate to the mass cultures for at least 2 days before the start of a test to eliminate animals injured during handling.

12.3.6 See 12.2.6 for procedures used to evaluate the health of cultures.

12.4 *Culturing Procedures for Chironomus tentans*:



Mean (+/- 2SD)

FIG. 1 Length and Relative Age of *Hyalella azteca* Collected by Sieving in Comparison with Length of Known-Age Organisms (118)

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12.4.1 The culturing methods described as follows are based on methods described in (**1**), (**8**), (**57**), (**191**), (**192**). If The objective of the study is to follow the test method for *C. tentans* outlined in 14.1.2, a 10-day test must be started with second- to third-instar larvae (about 10-day larvae Fig. 2). At a temperature of 23°C, larvae should develop to the third instar by 9 to 11 days after hatching (about 11 to 23 days post-oviposition). The instart of midges at the start of a test can be determined using head capsule width (see 14.1.2 and Table 13), weight, or length (average length of midge larvae should be about 4 to 6 mm or average dry weight should be about 0.08 to 0.23 mg/individual at the start of the test (**1**)). Historically, third-instar larvae were frequently referred to as second instar in the published literature. When larvae were measured daily, the C, tentans raised at 22 to 24°C were third instar, not second instar 9 to 11 days after hatching (**1**). A long-term test with *C. tentans* must be started with larvae less than 24-h old (see Section A7.3 for a description of an approach for obtaining *C. tentans* larvae less than 24-h old).

12.4.2 Both silica sand and shredded paper toweling have been used as substrates to culture *C. tentans*. Either substrate may be used if a healthy culture can be maintained. Greer (**192**) used sand or paper toweling to culture midges; however, sand was preferred due to the ease in removing larvae for testing. Sources of sand are listed in Section 7.

12.4.2.1 Paper towels are prepared according to a procedure adapted from (**194**). Plain white kitchen paper towels or brown towels are cut into strips. Cut toweling is loosely packed into a blender with culture water and blended for a few seconds. Small pieces should be available to the organism; blending for too long will result in a fine pulp that will not settle in culture tank. Blended towels can then be added directly to culture tanks eliminating any conditioning period for the substrate. A mass of the toweling sufficient to fill a 150-mL beaker is placed into a blender containing 1 L of deionized water, and blended for 30 s or until the strips are broken apart in the form of a pulp. The pulp is then sieved using a 710-µm sieve and thoroughly rinsed with deionized water to remove the shortest fibers.

12.4.2.2 Dry shredded paper toweling loosely packed into a 2-L beaker will provide sufficient substrate for about ten 19-L chambers (**191**). The shredded toweling placed in a 150-mL beaker produces enough substrate for one 19-L chamber. Additional substrate can be frozen in deionized water for later use.

12.4.3 Five egg cases will provide a sufficient number of organisms to start a new culture chamber. Egg cases should be held at 23°C in a glass beaker or crystallizing dish containing about 100 to 150 mL of culture water (temperature change should not exceed 2°C/day). Food is not added until the embryos start to hatch (in about 2 to 4 days at 23°C) to reduce the risk of oxygen depletion. A small amount of green algae (for example, a thin green layer) is added to the water when embryos start to hatch. When most of the larvae have left the egg case, 150 to 200 larvae should be placed into a culture chamber. Crowding of larvae will reduce growth. See 12.4.4.1 or 12.4.5.1 for a description of feeding rates. Larvae should reach the third instar by about 10 days after median hatch (about 12 to 14 days after the time the eggs were laid).

TABLE 13 ***Chironomus tentans*** **Instar and Head Capsule Widths (195)**

Instar	Days After Hatching	Mean, mm	Range, mm
First	1 to 4.4	0.10	0.09 to 0.13
Second	4.4 to 8.5	0.20	0.18 to 0.23
Third	8.5 to 12.5	0.38	0.33 to 0.45
Fourth	12.5–	0.67	0.63 to 0.71

12.4.4 *Chironomus tentans* are cultured in soft water at the USEPA in Duluth (USEPA, (**114**)) in glass aquaria (19.0-L capacity, 36- × 21- × 36-cm high). A water volume of about 6 to 8 L in these flow-through chambers can be maintained by drilling an overflow hole in one end 11 cm from the bottom. The top of the aquarium is covered with a mesh material to trap emergent adults. Pantyhose with the elasticized waist is positioned around the chamber top and the legs are cut off. Fiberglass-window screen glued to a glass-strip (about 2- to 3-cm wide) rectangle placed on top of each aquarium has also been used by Call et al. (**7**). About 200 to 300 mL of 40 mesh silica sand is placed in each chamber.

12.4.4.1 The stocking density of the number of *C. tentans* eggs should be about 600 eggs per 6 to 8 L of water. Dawson et al. (**386**) found that the cultures in 15 L aquaria and 7 L of water were self-regulating in density regardless of the initial number of eggs stocked in each tank. However, tanks with a higher initial stocking density (i.e., 1400 eggs) exhibited increased the time of peak adult emergence to 30 to 33 days while tanks with lower densities of 600 or 1000 eggs had peak emergence at 22 to 25 days after hatching.

12.4.4.2 Fish food flakes (i.e., Tetrafin®) are added to each culture chamber to provide a final food concentration of about 0.04 mg dry solids/mL of culture water. A stock suspension of the solids is blended with distilled water to form an initial slurry. It is then filtered through a 200 micron Nixtex screen and diluted with distilled water to form a 56 g dry solids/L final slurry (Annex A8). The larvae in each tank are fed 2.5 mL of slurry (140 mg of Tetrafin per day) from Day 0 to Day 7 and 5 mL of slurry (280 mg Tetrafin per day) from Day 8 on. Feeding is done after the water renewal process is completed. The stock suspension should be well mixed immediately before removing an aliquot for feeding. Each batch of food should be refrigerated and can be used for up to two weeks (Annex A8). Laboratories using static systems should develop lower feeding rates specific to thier systems

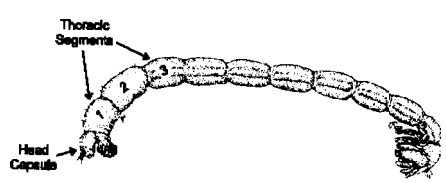

FIG. 2 *Chironomus tentans* **larvae. Note thoracic segments which are used to measure instars.**

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12.4.5 *Chironomus tentans* are cultured by Greer (**192**) in 5.7-L polyethylene cylindrical containers. The containers are modified by cutting a semicircle into the lid 17.75 cm across by 12.5 cm. Stainless steel screen (20 mesh/0.4 cm) is cut to size and melted to the plastic lid. The screen provides air exchange, retains emerging adults, and is a convenient way to observe the culture. Two holes about 0.05 cm in diameter are drilled through the uncut portion of the lid to provide access for an air line and to introduce food. The food access hole is closed with a No. 00 stopper. Greer (**192**) cultures midges under static conditions with moderate aeration and about 90 % of the water is replaced weekly. Each 5.7-L culture chamber contains about 3 L of water and about 25 mL of fine sand. Eight to ten chambers are used to maintain the culture.

12.4.5.1 Midges in each chamber are fed 2 mL/day of a 100 g/L suspension of fish food flakes on Tuesday, Wednesday, Thursday, Friday, and Sunday. A 2-mL *Chlorella* suspension of deactivated *Chlorella* suspension is added to each chamber on Saturday and Monday. The *Chlorella* suspension is prepared by adding 5 g of dry *Chlorella* powder/L of water. The mixture should be refrigerated and can be used for up to two weeks.

12.4.5.2 The water should be replaced more often if animals appear stressed (for example, at surface or pale color at the second instar) or if the water is cloudy. Water is replaced by first removing emergent adults with an aspirator. Any growth on the sides of the chamber should be brushed off before water is removed. Care should be taken not to pour or siphon out the larvae when removing the water. Larvae will typically stay near the bottom; however, a small mesh sieve or nylon net can be used to catch any larvae that float out. After the chambers have been cleaned, temperature-adjusted culture water is poured back into each chamber. The water should be added quickly to stir up the larvae. Using this procedure, the approximate size, number, and the general health of the culture can be observed.

12.4.6 Adult emergence will begin about three weeks after hatching at 23°C. Once adults begin to emerge, they can be gently siphoned into a dry aspirator flask on a daily basis. An aspirator can be made using a 250 or 500-mL Erlenmeyer flask, a two-hole stopper, some short sections of 0.25-in. glass tubing, and plastic tubing for collecting and providing suction (see Fig. 3). Adults should be aspirated with short inhalations to avoid injuring organisms. The mouthpiece on the aspirator should be replaced or disinfected between use. The sex ratio of the adults should be checked to ensure that a sufficient number of males are available for mating and fertilization. One male may fertilize more than one female. However, a ratio of one male to three females improves fertilization.

12.4.6.1 A mating and oviposition chamber may be prepared in several different ways (see Figs. 3 and 4). About 50 to 75 mL of culture water can be added to the aspiration flask in which the adults were collected (see Fig. 3; (**194**)). USEPA Duluth (**191**) used a 500-mL collecting flask (see Fig. 4), which includes a length of nylon screen[14] positioned vertically and extending into the water when water is added. The nylon screen[14] is used by the females to position themselves just above the water during oviposition. The two-hole stopper and tubing of the aspirator should be replaced by screened material or a cotton plug for good air exchange in the oviposition chamber.

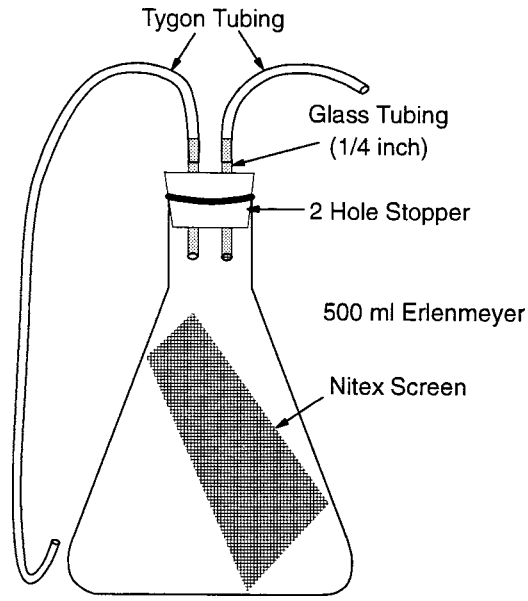

FIG. 3 Aspirators for Isolating Adult Midges

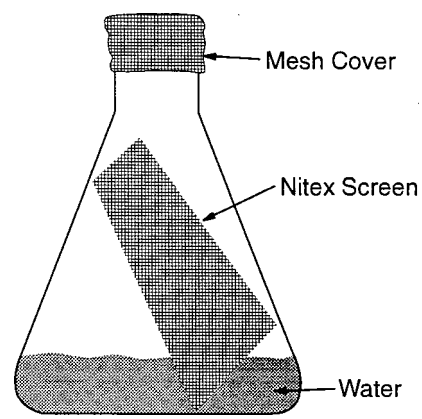

FIG. 4 Oviposition Chamber for Adult Midges

12.4.6.2 Greer (**192**) used an oviposition box to hold emergent adults which is constructed of a 5.7-L chamber with a 20-cm tall cylindrical chamber on top. The top chamber is constructed of stainless steel screen (35 mesh/2.54 cm) melted onto a plastic lid with a 17.75-cm hole. A 5-cm hole is cut into the side of the bottom chamber and a No. 11 stopper is used to close the hole. Egg cases are removed by first sliding a piece of plexiglass between the top and bottom chambers. Adult midges are then aspirated from the bottom chamber. The top chamber with plexiglass is removed from the bottom chamber and forceps are used to remove the egg cases. The top chamber is put back on top of the bottom chamber, the plexiglass is removed, and the aspirated adults are released from the aspirator into the chamber through the 5-cm hole.

12.4.7 About two to three weeks before the start of a test, 3 to 5 egg cases should be isolated for hatching using procedures outlined in 12.4.3.

[14] Coiled-wed material is available from 3-M, St. Paul, MN.

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12.4.8 Records should be kept on the time to first emergence and the success of emergence for each culture chamber. It is also desirable to monitor growth and head capsule width periodically in the cultures. See 12.2.6 for additional detail on procedures for evaluating the health of the cultures.

13. Procedure 1: Conducting a 10-day Sediment Toxicity Test with *Hyalella azteca*

13.1 *Introduction*:

13.1.1 *Hyalella azteca* (Saussure), Amphipoda, have many desirable characteristics of an ideal sediment toxicity testing organism including: relative sensitivity to contaminants associated with sediment, short generation time, contact with sediment, ease of culture in the laboratory, tolerance to varying physico-chemical characteristics of sediment, and their response has been evaluated in interlaboratory studies and has been confirmed with natural benthos populations (Table 1). Many investigators have successfully used *H. azteca* to evaluate the toxicity of freshwater sediments (for example, (**10, 17, 102, 118, 170, 196-198**)). *Hyalella azteca* can also be used to evaluate the toxicity of estuarine sediments (up to 15 ppt salinity; (**47, 143, 144, 187**)). Endpoints typically monitored in 10-day sediment toxicity tests with *H. azteca* include survival and growth.

13.1.2 General procedures for conducting sediment toxicity tests with *H. azteca* are outlined in Table 14, Annex A6 outlines additional procedures for evaluating sublethal effects of sediment-associated contaminants with *H. azteca* in long-term exposures. These general procedures can be used to conduct sediment toxicity tests with *H. azteca*. Methods outlined in Table 14 and in USEPA (**1**) were used for developing the specific test method described in 13.2 and Table 15 for conducting a 10-day toxicity test with *H. azteca*. The activity schedule in Table 16 and the test acceptability requirements in Table 17 are applicable to the methods described in Table 14 and Table 15.

13.1.3 Results of tests using procedures different from the procedures described in 13.2 and Table 15 may not be comparable to tests conducted with the procedures outlined in Table 14. Comparison of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with aquatic organisms. If tests are conducted with procedures different from the procedures described in these test methods, additional tests are required to determine comparability of results (see Section 1).

13.2 *Recommended Test Method for Conducting a 10-day Sediment Toxicity Test with Hyalella azteca*:

13.2.1 Recommended conditions for conducting a 10-day sediment toxicity test with *H. azteca* are summarized in Table 15. A general activity schedule is outlined in Table 16. Decisions concerning the various aspects of experimental design, such as the number of treatments, number of test chambers/treatment, and water quality characteristics should be based on the purpose of the test and the methods of data analysis (see Section 15). The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. When variability

TABLE 14 Summary of Testing Procedures Used to Evaluate the Toxicity of Whole Sediments with *Hyalella azteca*

Condition	Citation							
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
Temperature, °C	20	20	22	20–25	20–23	20–22	20	23
Light intensity (footcandles)	NR	25–50	NR	50–100	25–50	NR	50–100	50–100
Photoperiod	NR	16–8	16–8	16–8	16–8	16–8	16–8	16–8
Test chamber, mL	1000	1000	300	300	30–300	2500	250	300
Sediment volume, mL	200	200	100	40–50	5–100	~150	40	100
Overlying water volume, mL	800	100	175	160–200	20–150	~1350	200	175
Renewal rate of overlying water, additions/day	0	1–4	1–4	variable	0–2	0	static	2
Age of organisms, days	juvenile	juvenile	7–14	juvenile	7–14	0–7	juvenile	7–14
Size of organisms, mm	NR	1–2	NR	NR	1–2	NR	NR	1
Number of organisms/chamber	15	20	10	10	3–10	20	10	10
Number of replicate chambers/ treatment	NR	4	3	4	5–10	2	3	8
Food	RC	RC	YCT	RC	YCT, ML	TM	RC	YCT
Aeration	Yes	None	None	DO< 3	None	Yes	None	None
Overlying water	Natural	Natural	Natural	Natural	Reconstituted	Natural	Natural	Natural/ Reconstituted
Test duration, days	10	10–28	10	7	10	28	10	10
Endpoints	S	S,G,M	S	S	S	S,G	S	S,G
Test acceptability, survival, %	NR	80	80	80	80	NR	80	80

[A] Citations:
[1] Nebeker et al **()**
[2] Ingersoll and Nelson **(102)**
[3] Ankley et al **(4)**
[4] Burton et al **(195)**
[5] Winger and Lasier **(118)**
[6] Borgmann and Munawar **(194)**)
[7] Suedel and Rodgers **(18)**
[8] USEPA **(1)**

Conditions:
Food: YCT = yeast-cerophyll-trout chow, RC = Rabbit chow, TM = Fish food flakes,[12] ML = maple leaves.
Endpoints: S = survival, G = growth (length or weight), M = maturation.
NR: = not reported.

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TABLE 15 Test Conditions for Conducting a 10-day Sediment Toxicity Test with *Hyalella azteca*

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:	300-mL high-form lipless beaker
7. Sediment volume:	100 mL
8. Overlying water volume:	175 mL
9. Renewal of overlying water:	2 volume additions/day; continuous or intermittent (e.g., one volume addition every 12 h)
10. Age of organisms:	7- to 14-day old (within a 1- to 2-day range in age) at the start of the test
11. Number of organisms/chamber:	10
12. Number of replicate chambers/ treatment:	Depends on the objective of the test. Eight replicates are recommended for routine testing (see Section 15)
13. Feeding:	YCT food, fed 1.0 mL daily (1800 mg/L stock) to each test chamber. An earlier version of this standard (E 1706-95b) recommended a feeding level of 1.5 mL of YCT daily; however this feeding level was revised to 1.0 mL to be consistent with the feeding level in long-term tests with *H. azteca* (Annex A6).
14. Aeration:	None, unless dissolved oxygen in overlying water drops below 2.5 mg/L.
15. Overlying water:	Culture water, well water, surface water, site water, or reconstituted water
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, and ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily
18. Test duration:	10 d
19. Endpoints:	Survival and growth
20. Test acceptability:	Minimum mean control survival of 80 % and measurable growth of test organisms in the control sediment. See Table 17 for additional performance-based criteria.

TABLE 16 General Activity Schedule for Conducting a 10-day Sediment Toxicity Test with *Hyalella azteca* (modified from (6))

Day	Activity
-7	Separate known-age amphipods from the mass cultures and place in holding chambers. Begin preparing food for the test. There should be only a 1- to 2-day range in age of amphipods used to start the test.
-6 to -2	Feed and observe isolated amphipods, monitor water quality (e.g., temperature and dissolved oxygen).
-1	Feed and observe isolated amphipods, monitor water quality. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer 10 amphipods into each test chamber. Release organisms under the surface of the water. Add 1.0 mL of YCT into each test chamber. Archive 20 test organisms for length determination or 80 test organisms for dry weight determination. Observe behavior of test organisms.
1 to 8	Add 1.0 mL of YCT food to each test chamber. Measure temperature and dissolved oxygen. Observe behavior of test organisms.
9	Measure total water quality.
10	Measure temperature and dissolved oxygen. End the test by collecting the amphipods with a sieve. Count survivors and prepare organisms for dry weight or length measurements.

remains constant, the sensitivity of a test increases as the number of replicates increase.

13.2.2 The 10-day sediment toxicity test with *H. azteca* must be conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lx (Table 15). Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten 7- to 14-day old amphipods are used to start a test. The 10-day test should start with a narrow range in size or age of *H, azteca* (i.e., 1- to 2-day range in age) to reduce potential variability in growth at the end of a 10-day test (Section 12.3.1). The number of replicates/ treatment depends on the objective of the test. Eight replicates are recommended for routine testing (see Section 15). Amphipods in each test chamber are fed 1.0 mL of YCT food daily (**1**). The previous version of this standard (Test Method E 1706-95b) recommended a feeding level of 1.5 mL of YCT daily; however, this feeding level was revised to 1.0 mL to be consistent with the feeding level the in long-term test with *H. azteca* (Annex A6). Slight variation in feeding amount in the 10-day test with *C. tentans* did not influence the results of this test (Annex A9). Each chamber receives 2 volume additions/ day of overlying water. Benoit et al. (**128**) and Zumwalt et al. (**129**), Leppanen and Maier (**199**), and Wall et al. (**200**) describe water-renewal systems that can be used to deliver overlying water. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site at which sediment is collected. Requirements for test acceptability are summarized in Table 17.

13.3 *General Procedures*:

13.3.1 *Sediment into Test Chambers*—The day before the sediment test is started (Day -1) each sediment should be thoroughly homogenized and added to the test chambers (see 10.3.1). Sediment should be visually inspected to judge the extent of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components. If a quantitative measure of homogeneity is required, replicate subsamples should be taken from the sediment batch and analyze for TOC, chemical concentrations, and particle size.

13.3.1.1 Each test chamber should contain the same amount of sediment, determined either by volume or by weight. Overlying water is added to the chambers on Day -1 in a manner that minimizes suspension of sediment. This can be accomplished by gently pouring water along the sides of the chambers or by pouring water on to a baffle (for example, a circular piece of TFE-fluorocarbon with a handle attached) placed above the sediment to dissipate the force of the water. A test begins when the organisms are added to the test chambers (Day 0).

13.3.2 *Renewal of overlying water*—Renewal of overlying water is recommended during a test. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10 %. Hardness, alkalinity, and ammonia concentrations in the water above the sediment

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TABLE 17 Test Acceptability Requirements for a 10-day Sediment Toxicity Test with *Hyalella azteca*

A. It is recommended for conducting a 10-day test with *Hyalella azteca* that the following performance criteria be met:
1. Age of *H. azteca* at the start of the test must be between 7- to 14-days old. The test should start with a narrow narrow range in size or age of *H. azteca* (i.e., 1- to 2-day range in age) to reduce potential variability in growth at the end of a 10-day test (Section 12.3.1).
2. Average survival of *H. azteca* 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-day test (i.e., relative to organisms at the start of the test).
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 2.5 mg/L in the overlying water.

B. Performance-based criteria for culturing *H. azteca* include the following:
1. It may be desirable for laboratories to periodically perform 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms (Section 11.16.2). 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 track parental survival in the cultures and record this information using control charts if known-age cultures are maintained. Records should also be kept on the frequency of restarting cultures and the age of brood organisms.
3. Laboratories should record the following water-quality characteristics of the cultures at least quarterly pH, hardness, alkalinity, and ammonia. Dissolved oxygen in the cultures should be measured weekly. Temperature of the cultures should be recorded daily. If static cultures are used it may be desirable to measure water quality more frequently.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing oragnisms.
5. 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 Section 10.2.
3. All test chambers (and compartments) 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.

within a treatment, typically should not vary by more than 50 % during the test. Mount and Brungs (**126**) diluters have been modified for sediment testing and other automated water delivery systems have also been used (**102**, **127-129**, (**130**). Each water-delivery system should be calibrated before a test is started to verify that the system is functioning properly. Renewal of overlying water is started on Day -1 before the addition of test organisms and food on Day 0.

13.3.2.1 In water-renewal tests with one to four volume additions of overlying water/day, water quality characteristics generally remain similar to the inflowing water (**4**, **102**); however, in static tests, water quality may change profoundly during the exposure (**141**). For example, in static whole-sediment tests, the alkalinity, hardness, and conductivity of overlying water more than doubled in several treatments during a four-week exposure (**102**). Additionally, concentrations of metabolic products (for example, ammonia) may also increase during static exposures and these compounds can either be directly toxic to the test organisms or may contribute to the toxicity of the contaminants in the sediment. Furthermore, changes in water quality characteristics such as hardness may influence the toxicity of many inorganic (**201**) and organic (**202**) contaminants. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediment generally receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water.

13.3.3 *Acclimation*—Test organisms must be cultured and tested at the same temperature. Ideally, test organisms should be cultured in the same water that will be used in testing. However, acclimation of test organisms to the test water is not required. If test organisms are to be acclimated, they could be held for 2 h in a 50 to 50 mixture of culture water to overlying water, then for 2 h in a 25 to 75 mixture of culture water to overlying water, followed by a transfer into 100 % overlying water for 2 h (**102**).

13.3.4 *Placing Test Organisms in Test Chambers*—Amphipods should be introduced into the overlying water below the air-water interface. Test organisms can be pipeted directly into overlying water (**4**). Length should be measured on a subset of at least 20 organisms or weight should be measured on a subset of at least 80 organisms used to start the test. Test organisms should be handled as little as possible.

13.3.5 *Monitoring a Test*—All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

13.3.5.1 *Measurement of Overlying Water Quality Characteristics*—Conductivity, hardness, pH, alkalinity, dissolved oxygen, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to composite water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Water quality should be measured for each new batch of water prepared for the test.

(1) Dissolved oxygen should be measured daily and should be maintained at a minimum of 2.5 mg/L (Guide E 729). If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Accuracy

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can be used to maintain dissolved oxygen in the overlying water above 2.5 mg/L (i.e., about 1 bubble/second in the overlying water). Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

(2) Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath or the exposure chamber should be continuously monitored. 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.

13.3.6 *Feeding*—For each beaker, 1.0 mL of YCT is added from Day 0 to Day 9. Without addition of food, the test organisms may starve during exposures. However, the addition of the food may alter the availability of the contaminants in the sediment (**34, 99**). Furthermore, if too much food is added to the test chamber or if the mortality of test organisms is high, fungal or bacterial growth may develop on the sediment surface. Therefore, the amount of food added to the test chambers is kept to a minimum.

13.3.6.1 Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 2.5 mg/L during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration (**1**). If feeding is suspended in one treatment, it should be suspended in all treatments. Detailed records of feeding rates and the appearance of the sediment surface should be made daily.

13.3.7 *Ending a Test*—A consistent amount of time should be taken to examine sieved material for recovery of test organisms (for example, 5 min/replicate). Laboratories should demonstrate their personnel are able to recover an average of at least 90 % of the organisms from whole sediment. For example, test organisms could be added to control or test sediments and recovery could be determined after 1 h (**160**).

13.3.7.1 Any of the surviving amphipods in the water column or on the surface of the sediment can be pipeted from the beaker before sieving the sediment. Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. A #40 sieve (425 um mesh) can be used to remove amphipods from sediment. Alternatively, Kemble et al. (**17**) suggests sieving of sediment using the following procedure: (*1*) pour about half of the overlying water through a No. 50 (300-µm) U.S. Standard mesh sieve, (*2*) swirl the remaining water to suspend the upper 1 cm of sediment, (*3*) pour this slurry through the No. 50 mesh sieve and wash the contents of the sieve into an examination pan, *(4)* rinse the coarser sediment remaining in the test chamber through a No. 40 (425-µm) mesh sieve and wash the contents of this second sieve into an examination pan. Surviving test organisms can be removed from the two pans and counted. If length is to be measured (**102**), then the organisms can be preserved in 8 % sugar formalin solution or other substitutes for formalin (**203**). The sugar formalin solution is prepared adding 120 g of sucrose to 80 mL of formalin which is then brought to a volume of 1 L using deionized water. This stock solution is mixed with an equal volume of deionized water when used to preserve organisms.

13.3.8 *Test Data*—Survival and growth are measured at the end of the 10-day sediment toxicity test with *H. azteca*. Measuring growth may be a more sensitive toxicity endpoint compared to survival (**14, 17, 55**, (**204**). The duration of the 10-day test started with 7- to 14-day old amphipods is not long enough to determine sexual maturation or reproductive effects. The 42-day test (Annex A6) is designed to evaluate sublethal endpoints in sediment toxicity tests with *H. azteca*. See Section A6.4.5.2 for a discussion of measurement of length vs. dry weight in tests with *H. azteca*.

13.3.8.1 Amphipod body length (±0.1 mm) can be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface (Fig. 5). Ingersoll and Nelson (**102**) describe the use of a digitizing system and microscope to measure lengths of *H. azteca*. Kemble et al. (**17**) photographed invertebrates (at magnification of 3.5x) and measured length using a computer-interfaced digitizing tablet. Antennal segment number can also be used to estimate length or weight of amphipods (**1**). Wet or dry weight measurements have also been used to estimate growth of *H. azteca* (**1**). If test organisms are to be used for an evaluation of bioaccumulation, it is not advisable to dry the sample before conducting the residue analysis. If conversion from wet weight to dry weight is necessary, aliquots of organisms can be weighed to establish wet to dry weight conversion factors. A consistent procedure should be used to remove the excess water from the organisms before measuring wet weight.

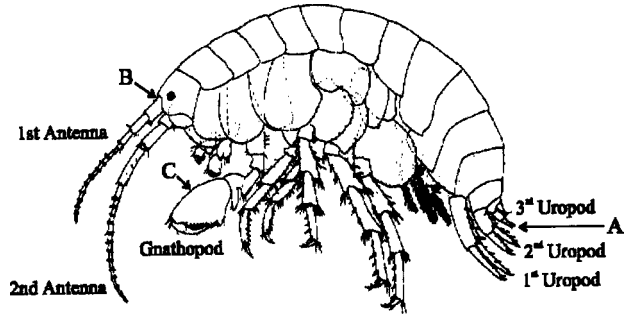

FIG. 5 *Hyalella azteca*. **A: denotes the uropods, B: denotes the base of the first antennae. C: denotes the gnathopod used for grasing females. Measurement of length is meade from base of the 3rd uropod (A) to (B). Females are recognized by the presence of egg cases or the absence of an enlarged gnathopod.**

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