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Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates[1]

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1. Scope*

1.1 This test method covers procedures for testing estuarine or marine 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. General guidance is presented in Sections 1 – 15 for conducting sediment toxicity tests with estuarine or marine amphipods. Specific guidance for conducting 10-d sediment toxicity tests with estuarine or marine amphipods is outlined in Annex A1 and specific guidance for conducting 28-d sediment toxicity tests with *Leptocheirus plumulosus* is outlined in Annex A2.

1.2 Procedures are described for testing estuarine or marine amphipod crustaceans in 10-d laboratory exposures to evaluate the toxicity of contaminants associated with whole sediments (Annex A1; USEPA 1994a (**1**)). Sediments may be collected from the field or spiked with compounds in the laboratory. A toxicity method is outlined for four species of estuarine or marine sediment-burrowing amphipods found within United States coastal waters. The species are *Ampelisca abdita*, a marine species that inhabits marine and mesohaline portions of the Atlantic coast, the Gulf of Mexico, and San Francisco Bay; *Eohaustorius estuarius*, a Pacific coast estuarine species; *Leptocheirus plumulosus*, an Atlantic coast estuarine species; and *Rhepoxynius abronius*, a Pacific coast marine species. Generally, the method described may be applied to all four species, although acclimation procedures and some test conditions (that is, temperature and salinity) will be species-specific (Sections 12 and Annex A1). The toxicity test is conducted in 1-L glass chambers containing 175 mL of sediment and 775 mL of overlying seawater. Exposure is static (that is, water is not renewed), and the animals are not fed over the 10-d exposure period. The endpoint in the toxicity test is survival with reburial of surviving amphipods as an additional measurement that can be used as an endpoint for some of the test species (for *R. abronius* and *E. estuarius*). Performance criteria established for this test include the average survival of amphipods in negative control treatment must be greater than or equal to 90 %. Procedures are described for use with sediments with pore-water salinity ranging from >0 ‰ to fully marine.

1.3 A procedure is also described for determining the chronic toxicity of contaminants associated with whole sediments with the amphipod *Leptocheirus plumulosus* in laboratory exposures (Annex A2; USEPA-USACE 2001(**2**)). The toxicity test is conducted for 28 d in 1-L glass chambers containing 175 mL of sediment and about 775 mL of overlying water. Test temperature is 25° ± 2°C, and the recommended overlying water salinity is 5 ‰ ± 2 ‰ (for test sediment with pore water at 1 ‰ to 10 ‰) or 20 ‰ ± 2 ‰ (for test sediment with pore water >10 ‰). Four hundred millilitres of overlying water is renewed three times per week, at which times test organisms are fed. The endpoints in the toxicity test are survival, growth, and reproduction of amphipods. Performance criteria established for this test include the average survival of amphipods in negative control treatment must be greater than or equal to 80 % and there must be measurable growth and reproduction in all replicates of the negative control treatment. This test is applicable for use with sediments from oligohaline to fully marine environments, with a silt content greater than 5 % and a clay content less than 85 %.

1.4 A salinity of 5 or 20 ‰ is recommended for routine application of 28-d test with *L. plumulosus* (Annex A2; USEPA-USACE 2001 (**2**)) and a salinity of 20 ‰ is recommended for routine application of the 10-d test with *E. estuarius* or *L. plumulosus* (Annex A1). However, the salinity of the overlying water for tests with these two species can be adjusted to a specific salinity of interest (for example, salinity representative of site of interest or the objective of the study may be to evaluate the influence of salinity on the bioavailability of chemicals in sediment). More importantly, the salinity tested must be within the tolerance range of the test organisms (as outlined in Annex A1 and Annex A2). If tests are conducted with procedures different from those described in 1.3 or in Table A1.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (1.10). If there is not a

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

TABLE 1 Rating of Selection Criteria for Estuarine or Marine Amphipod Sediment Toxicity Testing A "+" or "–" Rating Indicates a Positive or Negative Attribute

Criterion	*Ampelisca abdita*	*Eohaustorius estuarius*	*Leptocheirus plumulosus*	*Rhepoxynius abronius*
Relative sensitivity toxicity data base	+	+	+	+
Round-robin studies conducted	+	+	+	+
Contact with sediment	+	+	+	+
Laboratory culture	+/-	-	+	-
Taxonomic identification	+	+	+	+
Ecological importance	+	+	+	+
Geographical distribution	ATL, PAC, GOM	PAC	ATL	PAC
Sediment physicochemical tolerance	+	+	+	+
Response confirmed with benthos populations	+	+[A]	+	+
Peer reviewed	+	+	+	+
Endpoints monitored	Survival	Survival, reburial	Survival	Survival, reburial

[A] Anderson et al. (2001 (**14**)).

ATL = Atlantic Coast, PAC = Pacific Coast, GOM= Gulf of Mexico

need to make comparisons among studies, then the test could be conducted just at a selected salinity for the sediment of interest.

1.5 Future revisions of this standard may include additional annexes describing whole-sediment toxicity tests with other groups of estuarine or marine invertebrates (for example, information presented in Guide E1611 on sediment testing with polychaetes could be added as an annex to future revisions to this standard). Future editions to this standard may also include methods for conducting the toxicity tests in smaller chambers with less sediment (Ho et al. 2000 (**3**), Ferretti et al. 2002 (**4**)).

1.6 Procedures outlined in this standard are based primarily on procedures described in the USEPA (1994a (**1**)), USEPA-USACE (2001(**2**)), Test Method E1706, and Guides E1391, E1525, E1688, Environment Canada (1992 (**5**)), DeWitt et al. (1992a (**6**); 1997a (**7**)), Emery et al. (1997 (**8**)), and Emery and Moore (1996 (**9**)), Swartz et al. (1985 (**10**)), DeWitt et al. (1989 (**11**)), Scott and Redmond (1989 (**12**)), and Schlekat et al. (1992 (**13**)).

1.7 Additional sediment toxicity research and methods development are now in progress to *(1)* refine sediment spiking procedures, *(2)* refine sediment dilution procedures, *(3)* refine sediment Toxicity Identification Evaluation (TIE) procedures, *(4)* produce additional data on confirmation of responses in laboratory tests with natural populations of benthic organisms (that is, field validation studies), and *(5)* evaluate relative sensitivity of endpoints measured in 10- and 28-d toxicity tests using estuarine or marine amphipods. This information will be described in future editions of this standard.

1.8 Although standard procedures are described in Annex A2 of this standard for conducting chronic sediment tests with *L. plumulosus*, further investigation of certain issues could aid in the interpretation of test results. Some of these issues include further investigation to evaluate the relative toxicological sensitivity of the lethal and sublethal endpoints to a wide variety of chemicals spiked in sediment and to mixtures of chemicals in sediments from contamination gradients in the field (USEPA-USACE 2001 (**2**)). Additional research is needed to evaluate the ability of the lethal and sublethal endpoints to estimate the responses of populations and communities of benthic invertebrates to contaminated sediments. Research is also needed to link the toxicity test endpoints to a field-validated population model of *L. plumulosus* that would then generate estimates of population-level responses of the amphipod to test sediments and thereby provide additional ecologically relevant interpretive guidance for the laboratory toxicity test.

1.9 This standard outlines specific test methods for evaluating the toxicity of sediments with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius*. While standard procedures are described in this standard, further investigation of certain issues could aid in the interpretation of test results. Some of these issues include the effect of shipping on organism sensitivity, additional performance criteria for organism health, sensitivity of various populations of the same test species, and confirmation of responses in laboratory tests with natural benthos populations.

1.10 General procedures described in this standard might be useful for conducting tests with other estuarine or marine organisms (for example, *Corophium spp.*, *Grandidierella japonica*, *Lepidactylus dytiscus*, *Streblospio benedicti*), although modifications may be necessary. 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.

1.11 *Selection of Toxicity Testing Organisms:*

1.11.1 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 E1525). Ideally, a test organism should: *(1)* have a toxicological database demonstrating relative sensitivity to a range of

contaminants of interest in sediment, *(2)* have a database for interlaboratory comparisons of procedures (for example, round-robin studies), *(3)* be in direct contact with sediment, *(4)* be readily available from culture or through 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 (Guide E1525). Methods utilizing selected organisms should also be *(11)* peer reviewed (for example, journal articles) and *(12)* confirmed with responses with natural populations of benthic organisms.

1.11.2 Of these criteria (Table 1), a database demonstrating relative sensitivity to contaminants, contact with sediment, ease of culture in the laboratory or availability for field-collection, ease of handling in the laboratory, tolerance to varying sediment physico-chemical characteristics, and confirmation with responses with natural benthic populations were the primary criteria used for selecting *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* for the current edition of this standard for 10-d sediment tests (Annex A1). The species chosen for this method are intimately associated with sediment, due to their tube- dwelling or free-burrowing, and sediment ingesting nature. Amphipods have been used extensively to test the toxicity of marine, estuarine, and freshwater sediments (Swartz et al., 1985 (**10**); DeWitt et al., 1989 (**11**); Scott and Redmond, 1989 (**12**); DeWitt et al., 1992a (**6**); Schlekat et al., 1992 (**13**)). The selection of test species for this standard followed the consensus of experts in the field of sediment toxicology who participated in a workshop entitled "Testing Issues for Freshwater and Marine Sediments". The workshop was sponsored by USEPA Office of Water, Office of Science and Technology, and Office of Research and Development, and was held in Washington, D.C. from 16-18 September 1992 (USEPA, 1992 (**15**)). Of the candidate species discussed at the workshop, *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* best fulfilled the selection criteria, and presented the availability of a combination of one estuarine and one marine species each for both the Atlantic (the estuarine *L. plumulosus* and the marine *A. abdita*) and Pacific (the estuarine *E. estuarius* and the marine *R. abronius*) coasts. *Ampelisca abdita* is also native to portions of the Gulf of Mexico and San Francisco Bay. Many other organisms that might be appropriate for sediment testing do not now meet these selection criteria because little emphasis has been placed on developing standardized testing procedures for benthic organisms. For example, a fifth species, *Grandidierella japonica* was not selected because workshop participants felt that the use of this species was not sufficiently broad to warrant standardization of the method. Environment Canada (1992 (**5**)) has recommended the use of the following amphipod species for sediment toxicity testing: *Amphiporeia virginiana*, *Corophium volutator*, *Eohaustorius washingtonianus*, *Foxiphalus xiximeus*, and *Leptocheirus pinguis*. A database similar to those available for *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* must be developed in order for these and other organisms to be included in future editions of this standard.

1.11.3 The primary criterion used for selecting *L. plumulosus* for chronic testing of sediments was that this species is found in both oligohaline and mesohaline regions of estuaries on the East Coast of the United States and is tolerant to a wide range of sediment grain size distribution (USEPA-USACE 2001 (**2**), Annex Annex A2). This species is easily cultured in the laboratory and has a relatively short generation time (that is, about 24 d at 23°C, DeWitt et al. 1992a(**6**)) that makes this species adaptable to chronic testing (Section 12).

1.11.4 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. Several studies have evaluated the sensitivities of *A. abdita*, *E. estuarius*, *L. plumulosus*, or *R. abronius*, either relative to one another, or to other commonly tested estuarine or marine species. For example, the sensitivity of marine amphipods was compared to other species that were used in generating saltwater Water Quality Criteria. Seven amphipod genera, including *Ampelisca abdita* and *Rhepoxynius abronius*, were among the test species used to generate saltwater Water Quality Criteria for 12 chemicals. Acute amphipod toxicity data from 4-d water-only tests for each of the 12 chemicals was compared to data for *(1)* all other species, *(2)* other benthic species, and *(3)* other infaunal species. Amphipods were generally of median sensitivity for each comparison. The average percentile rank of amphipods among all species tested was 57 %; among all benthic species, 56 %; and, among all infaunal species, 54 %. Thus, amphipods are not uniquely sensitive relative to all species, benthic species, or even infaunal species (USEPA 1994a (**1**)). Additional research may be warranted to develop tests using species that are consistently more sensitive than amphipods, thereby offering protection to less sensitive groups.

1.11.5 Williams et al. (1986 (**16**)) compared the sensitivity of the *R. abronius* 10-d whole sediment test, the oyster embryo (*Crassostrea gigas*) 48-h abnormality test, and the bacterium (*Vibrio fisheri*) 1-h luminescence inhibition test (that is, the Microtox[2] test) to sediments collected from 46 contaminated sites in Commencement Bay, WA. *Rhepoxynius abronius* were exposed to whole sediment, while the oyster and bacterium tests were conducted with sediment elutriates and extracts, respectfully. Microtox[2] was the most sensitive test, with 63 % of the sites eliciting significant inhibition of luminescence. Significant mortality of *R. abronius* was observed in 40 % of test sediments, and oyster abnormality occurred in 35 % of sediment elutriates. Complete concordance (that is, sediments that were either toxic or not-toxic in all three tests) was observed in 41 % of the sediments. Possible sources for the lack of concordance at other sites include interspecific differences in sensitivity among test organisms, heterogeneity in contaminant types associated with test sediments, and differences in routes of exposure inherent in each toxicity test. These

[2] Microtox is a trademark of Strategic Diagnostics Inc. 111 Pencader Drive Newark, Delaware 19702-3322.

results highlight the importance of using multiple assays when performing sediment assessments.

1.11.6 Several studies have compared the sensitivity of combinations of the four amphipods to sediment contaminants. For example, there are several comparisons between *A. abdita* and *R. abronius*, between *E. estuarius* and *R. abronius*, and between *A. abdita* and *L. plumulosus*. There are fewer examples of direct comparisons between *E. estuarius* and *L. plumulosus*, and no examples comparing *L. plumulosus* and *R. abronius*. There is some overlap in relative sensitivity from comparison to comparison within each species combination, which appears to indicate that all four species are within the same range of relative sensitivity to contaminated sediments.

1.11.6.1 Word et al. (1989 (**17**)) compared the sensitivity of *A. abdita* and *R. abronius* to contaminated sediments in a series of experiments. Both species were tested at 15°C. Experiments were designed to compare the response of the organism rather than to provide a comparison of the sensitivity of the methods (that is, *Ampelisca abdita* would normally be tested at 20°C). Sediments collected from Oakland Harbor, CA, were used for the comparisons. Twenty-six sediments were tested in one comparison, while 5 were tested in the other. Analysis of results using Kruskal Wallace rank sum test for both experiments demonstrated that *R. abronius* exhibited greater sensitivity to the sediments than *A. abdita* at 15°C. Long and Buchman (1989 (**18**)) also compared the sensitivity of *A. abdita* and *R. abronius* to sediments from Oakland Harbor, CA. They also determined that *A. abdita* showed less sensitivity than *R. abronius*, but they also showed that *A. abdita* was less sensitive to sediment grain size factors than *R. abronius*.

1.11.6.2 DeWitt et al. (1989 (**11**)) compared the sensitivity of *E. estuarius* and *R. abronius* to sediment spiked with fluoranthene and field-collected sediment from industrial waterways in Puget Sound, WA, in 10-d tests, and to aqueous cadmium ($CdCl_2$) in a 4-d water-only test. The sensitivity of *E. estuarius* was from two (to spiked-spiked sediment) to seven (to one Puget Sound, WA, sediment) times less sensitive than *R. abronius* in sediment tests, and ten times less sensitive to $CdCl_2$ in the water-only test. These results are supported by the findings of Pastorok and Becker (1990 (**19**)) who found the acute sensitivity of *E. estuarius* and *R. abronius* to be generally comparable to each other, and both were more sensitive than *Neanthes arenaceodentata* (survival and biomass endpoints), *Panope generosa* (survival), and *Dendraster excentricus* (survival).

1.11.6.3 *Leptocheirus plumulosus* was as sensitive as the freshwater amphipod *Hyalella azteca* to an artificially created gradient of sediment contamination when the latter was acclimated to oligohaline salinity (that is, 6 ‰ ; McGee et al., 1993 (**20**)). DeWitt et al. (1992b (**21**)) compared the sensitivity of *L. plumulosus* with three other amphipod species, two mollusks, and one polychaete to highly contaminated sediment collected from Baltimore Harbor, MD, that was serially diluted with clean sediment. *Leptocheirus plumulosus* was more sensitive than the amphipods *Hyalella azteca* and *Lepidactylus dytiscus* and exhibited equal sensitivity with *E. estuarius*. Schlekat et al. (1995 (**22**)) describe the results of an interlaboratory comparison of 10-d tests with *A. abdita*, *L. plumulosus* and *E. estuarius* using dilutions of sediments collected from Black Rock Harbor, CT. There was strong agreement among species and laboratories in the ranking of sediment toxicity and the ability to discriminate between toxic and non-toxic sediments.

1.11.6.4 Hartwell et al. (2000 (**23**)) evaluated the response of *Leptocheirus plumulosus* (10-d survival or growth) to the response of the amphipod *Lepidactylus dytiscus* (10-d survival or growth), the polychaete *Streblospio benedicti* (10-d survival or growth), and lettuce germination (Lactuca sativa in 3-d exposure) and observed that *L. plumulosus* was relatively insensitive compared to the response of either L. dytiscus or S. benedicti in exposures to 4 sediments with elevated metal concentrations.

1.11.6.5 Ammonia is a naturally occurring compound in marine sediment that results from the degradation of organic debris. Interstitial ammonia concentrations in test sediment can range from <1 mg/L to in excess of 400 mg/L (Word et al., 1997 (**24**)). Some benthic infauna show toxicity to ammonia at concentrations of about 20 mg/L (Kohn et al., 1994 (**25**)). Based on water-only and spiked-sediment experiments with ammonia, threshold limits for test initiation and termination have been established for the *L. plumulosus* chronic test. Smaller (younger) individuals are more sensitive to ammonia than larger (older) individuals (DeWitt et al., 1997a(**7**), b (**26**). Results of a 28-d test indicated that neonates can tolerate very high levels of pore-water ammonia (>300 mg/L total ammonia) for short periods of time with no apparent long-term effects (Moore et al., 1997 (**27**)). It is not surprising *L. plumulosus* has a high tolerance for ammonia given that these amphipods are often found in organic rich sediments in which diagenesis can result in elevated pore-water ammonia concentrations. Insensitivity to ammonia by *L. plumulosus* should not be construed as an indicator of the sensitivity of the *L. plumulosus* sediment toxicity test to other chemicals of concern.

1.11.7 Limited comparative data is available for concurrent water-only exposures of all four species in single-chemical tests. Studies that do exist generally show that no one species is consistently the most sensitive.

1.11.7.1 The relative sensitivity of the four amphipod species to ammonia was determined in ten-d water only toxicity tests in order to aid interpretation of results of tests on sediments where this toxicant is present (USEPA 1994a (**1**)). These tests were static exposures that were generally conducted under conditions (for example, salinity, photoperiod) similar to those used for standard 10-d sediment tests. Departures from standard conditions included the absence of sediment and a test temperature of 20°C for *L. plumulosus*, rather than 25°C as dictated in this standard. Sensitivity to total ammonia increased with increasing pH for all four species. The rank sensitivity was *R. abronius* = *A. abdita* > *E. estuarius* > *L. plumulosus*. A similar study by Kohn et al. (1994 (**25**)) showed a similar but slightly different relative sensitivity to ammonia with *A. abdita* > *R. abronius* = *L. plumulosus* > *E. estuarius*.

1.11.7.2 Cadmium chloride has been a common reference toxicant for all four species in 4-d exposures. DeWitt et al. (1992a (**6**)) reports the rank sensitivity as *R. abronius* > *A. abdita* > *L. plumulosus* > *E. estuarius* at a common temperature and salinity of 15°C and 28 ‰ . A series of 4-d exposures

to cadmium that were conducted at species-specific temperatures and salinities showed the following rank sensitivity: *A. abdita* = *L. plumulosus* = *R. abronius* > *E. estuarius* (USEPA 1994a (**1**)).

1.11.7.3 Relative species sensitivity frequently varies among contaminants; consequently, a battery of tests including organisms representing different trophic levels may be needed to assess sediment quality (Craig, 1984 (**28**); Williams et al. 1986 (**16**); Long et al., 1990 (**29**); Ingersoll et al., 1990 (**30**); Burton and Ingersoll, 1994 (**31**)). For example, Reish (1988 (**32**)) 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.

1.11.8 The sensitivity of an organism is related to route of exposure and biochemical response to contaminants. Sediment-dwelling organisms can receive exposure 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 contaminants from sediment. Benthic invertebrates often selectively consume different particle sizes (Harkey et al. 1994 (**33**)) or particles with higher organic carbon concentrations which may have higher contaminant concentrations. Grazers and other collector-gatherers that feed on aufwuchs and detritus may receive most of their body burden directly from materials attached to sediment or from actual sediment ingestion. In some amphipods (Landrum, 1989 (**34**)) and clams (Boese et al., 1990 (**35**)) uptake through the gut can exceed uptake across the gills for certain hydrophobic compounds. Organisms in direct contact with sediment may also accumulate contaminants by direct adsorption to the body wall or by absorption through the integument (Knezovich et al. 1987 (**36**)).

1.11.9 Despite the potential complexities in estimating the dose that an animal receives from sediment, the toxicity and bioaccumulation of many contaminants in sediment such as Kepone®, fluoranthene, organochlorines, and metals have been correlated with either the concentration of these chemicals in interstitial water or in the case of non-ionic organic chemicals, concentrations in sediment on an organic carbon normalized basis (Di Toro et al. 1990 (**37**); Di Toro et al. 1991 (**38**)). The relative importance of whole sediment and interstitial water routes of exposure depends on the test organism and the specific contaminant (Knezovich et al. 1987 (**36**)). 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.

1.11.10 The use of *A. abdita*, *E. estuarius*, *R. abronius*, and *L. plumulosus* in laboratory toxicity studies has been field validated with natural populations of benthic organisms (Swartz et al. 1994 (**39**) and Anderson et al. 2001 (**14**) for *E. estuarius*, Swartz et al. 1982 (**40**) and Anderson et al. 2001 (**14**) for *R. abronius*, McGee et al. 1999 (**41**) and McGee and Fisher 1999 (**42**) for *L. plumulosus*).

1.11.10.1 Data from USEPA Office of Research and Development's Environmental Monitoring and Assessment program were examined to evaluate the relationship between survival of *Ampelisca abdita* in sediment toxicity tests and the presence of amphipods, particularly ampeliscids, in field samples. Over 200 sediment samples from two years of sampling in the Virginian Province (Cape Cod, MA, to Cape Henry, VA) were available for comparing synchronous measurements of *A. abdita* survival in toxicity tests to benthic community enumeration. Although species of this genus were among the more frequently occurring taxa in these samples, ampeliscids were totally absent from stations that exhibited *A. abdita* test survival <60 % of that in control samples. Additionally, ampeliscids were found in very low densities at stations with amphipod test survival between 60 and 80 % (USEPA 1994a (**1**)). These data indicate that tests with this species are predictive of contaminant effects on sensitive species under natural conditions.

1.11.10.2 Swartz et al. (1982 (**40**)) compared sensitivity of *R. abronius* to sediment collected from sites in Commencement Bay, WA, to benthic community structure at each site. Mortality of *R. abronius* was negatively correlated with amphipod density, and phoxocephalid amphipods were ubiquitously absent from the most contaminated areas.

1.11.10.3 Sediment toxicity to amphipods in 10-d toxicity tests, field contamination, and field abundance of benthic amphipods were examined along a sediment contamination gradient of DDT (Swartz et al. 1994 (**39**)). Survival of *E. estuarius* and *R. abronius* in laboratory toxicity tests was positively correlated to abundance of amphipods in the field and along with the survival of *H. azteca*, was negatively correlated to DDT concentrations. The threshold for 10-d sediment toxicity in laboratory studies was about 300 ug DDT (+metabolites)/g organic carbon. The threshold for abundance of amphipods in the field was about 100 ug DDT (+metabolites)/g organic carbon. Therefore, correlations between toxicity, contamination, and biology indicate that acute 10-d sediment toxicity tests can provide reliable evidence of biologically adverse sediment contamination in the field.

1.11.10.4 As part of a comprehensive sediment quality assessment in Baltimore Harbor, MD, McGee et al. (1999 (**41**)) conducted 10-d toxicity tests with *L. plumulosus*. Negative relationships were detected between amphipod survival and concentrations of select sediment-associated contaminants, whereas a very strong positive association existed between survival in laboratory exposures and field density of *L. plumulosus* at test sites. A field validation study of the 10- and 28-d *L. plumulosus* tests by McGee and Fisher (1999 (**42**)) in Baltimore Harbor, also indicated good agreement between acute toxicity, sediment associated contaminants and responses of the *in situ* benthic community. In this study, the chronic 28-d test was less sensitive to sediment contamination than the acute 10-d test; however, the feeding regime used in this evaluation is different than the one currently recommended in Annex A2 and may have influenced the test results. Field validation studies with the revised 28-d test outlined in Annex A2 have not been conducted.

1.12 *Chronic Sediment Methods with Leptocheirus plumulosus:*

1.12.1 Most standard whole sediment toxicity tests have been developed to produce a lethality endpoint (survival/mortality) with potential for a sublethal endpoint (reburial) in some species (USEPA 1994a (**1**), USEPA-USACE 2001 (**2**)). Methods that measure sublethal effects have not been available or have not been routinely used to evaluate sediment toxicity in marine or estuarine sediments (Scott and Redmond, 1989 (**12**); Green and Chandler, 1996 (**43**); Levin et al., 1996 (**44**); Ciarelli et al., 1998 (**45**); Meador and Rice, 2001 (**46**)). Most assessments of contaminated sediment rely on short-term lethality tests (for example, ≤10 d; USEPA-USACE, 1991 (**47**); 1998 (**48**)). Short-term lethality tests are useful in identifying "hot spots" of sediment contamination, but might not be sensitive enough to evaluate moderately contaminated areas. However, 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 (Ingersoll et al., 1998 (**49**); Kemble et al., 1994 (**50**); McGee et al., 1995 (**51**); Scott, 1989 (**52**)). The 28-d toxicity test with *Leptocheirus plumulosus* has two sublethal endpoints: growth and reproduction. These sublethal endpoints have potential to exhibit a toxic response from chemicals that otherwise might not cause acute effects or significant mortality in a test. Sublethal response to chronic exposure is also valuable for population modeling of contaminant effects. These data can be used for population-level risk assessments of benthic pollutant effects.

1.12.2 An evaluation of the distribution of *L. plumulosus* in Chesapeake Bay indicates that its distribution is negatively correlated with the degree of sediment contamination (Pfitzenmeyer, 1975 (**53**); Reinharz, 1981 (**54**)). A field validation study of the 10- and 28-d *L. plumulosus* tests by McGee and Fisher (1999 (**42**)) in Baltimore Harbor, indicated good agreement between acute toxicity, sediment associated contaminants and responses of the *in situ* benthic community. In this study, the chronic 28-d test was less sensitive to sediment contamination than the acute 10-d test and therefore had a poorer association between sediment contaminants and benthic community health. It should be noted that the feeding regime used in this evaluation is different than the one currently recommended in Annex A2 and may have influenced the test results. Field validation studies with the revised 28-d test have not been conducted.

1.13 *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.

1.14 This standard is arranged as follows:

1.15 *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:*[3]

D1129 Terminology Relating to Water

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E29 Practice for Using Significant Digits in Test Data to Determine Conformance with Specifications

E105 Practice for Probability Sampling of Materials

E122 Practice for Calculating Sample Size to Estimate, With Specified Precision, the Average for a Characteristic of a Lot or Process

E141 Practice for Acceptance of Evidence Based on the Results of Probability Sampling

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

E178 Practice for Dealing With Outlying Observations

E456 Terminology Relating to Quality and Statistics

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

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes

E1325 Terminology Relating to Design of Experiments

E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

E1402 Guide for Sampling Design

E1525 Guide for Designing Biological Tests with Sediments

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

E1611 Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids
E1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates
E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates
E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines
E1850 Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests
IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

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 E729 and E1241 and Terminology E943 and D1129. For an explanation of units and symbols, refer to IEEE/ASTM SI 10IEEE/ASTM SI 10.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *clean, n*—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, n*—the ratio of weight or volume of test material(s) to the weight or volume of sediment.

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

3.3.4 *control sediment, n*—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, n*—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, n*—mixtures of materials used to mimic the physical components of a natural sediment.

3.3.7 *IC50, n*—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, n*— water occupying space between sediment or soil particles.

3.3.9 *LC50, n*—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), n*—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), n—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, n*—the water placed over sediment in a test chamber during a test.

3.3.13 *reference sediment, n*—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, n*—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, n*—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, n*—a sediment to which a material has been added for experimental purposes.

3.3.17 *whole sediment, n*—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 estuarine or marine amphipod crustaceans in the 10-d laboratory exposures to evaluate the toxicity of contaminants associated with whole sediments. Sediments may be collected from the field or spiked with compounds in the laboratory. A toxicity method is outlined for four species of estuarine or marine sediment-burrowing amphipods found within United States coastal waters. The species are *Ampelisca abdita*, a marine species that inhabits marine and mesohaline portions of the Atlantic coast, the Gulf of Mexico, and San Francisco Bay; *Eohaustorius estuarius*, a Pacific coast estuarine species; *Leptocheirus plumulosus*, an Atlantic coast estuarine species; and *Rhepoxynius abronius*, a Pacific coast marine species. Generally, the method described may be applied to all four species, although acclimation procedures and some test conditions (that is, temperature and salinity) will be species-specific (Sections 10 and 11). The toxicity test is conducted in 1-L glass chambers containing 175 mL of sediment and 775 mL of overlying seawater. Exposure is static (that is, water is not renewed), and the animals are not fed over the 10-d exposure period. The endpoint in the toxicity test is survival with reburial of surviving amphipods as an additional measurement that can be used as an endpoint for some of the test species (for *R. abronius* and *E. estuarius*). Performance criteria established for this test include the average survival of amphipods in negative control treatment must be greater than or equal to 90 %. Procedures are described for use with sediments with pore-water salinity ranging from >0 % to fully marine.

4.2 A procedure is also described for determining the chronic toxicity of contaminants associated with whole sediments with the amphipod *Leptocheirus plumulosus* in laboratory exposures (USEPA-USACE 2001 (**2**)). The toxicity test is conducted for 28 d in 1-L glass chambers containing 175 mL of sediment and about 775 mL of overlying seawater. Four hundred millilitres of overlying water is renewed three times per week, at which time test organisms are fed. Tests are initiated with neonate amphipods that mature and reproduce during the 28-d test period. The endpoints in the 28-d toxicity test are survival, growth rate, and reproduction of amphipods. Survival is calculated as the percentage of newly born (neonate) amphipods at test initiation that survive as adults at test termination. Growth rate is calculated as the mean dry weight gain per day per adult amphipod surviving at test termination. Reproduction is calculated as the number of offspring per surviving adult. This test is applicable for use with sediment having pore-water salinity ranging from 1 ‰ to 35 ‰ . Typically, endpoint selection for new toxicity tests is generally guided by methodologies for related toxicity tests (Gray et al., 1998 (**55**)). Sediment toxicity tests using macroinvertebrates often incorporate survival and growth endpoints (Ingersoll, 1995 (**56**)). Gray et al. (1998 (**55**)) recommend optimal endpoint measures for the *L. plumulosus* sediment toxicity test based on four criteria: relevance of each measure to its respective endpoint; signal-to-noise ratio (the ratio between the response to stressor and the normal variation in the response variable); redundancy to other measures of the same endpoint; and cost of labor, training, and equipment. Signal-to-noise ratios are independent of experiment design considerations (that is, Type I and Type II errors, and sample size) and are positively correlated with power (Gray et al., 1998 (**55**)).

4.3 *Experimental Design*—The following section is a general summary of experimental design. See Section 13 for additional detail.

4.3.1 *Control and Reference Sediment:*

4.3.1.1 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 Ankley and Thomas, 1992 (**57**). Comparing test sediments to control sediments is a measure of the toxicity of a test sediment beyond inevitable background contamination and organism health Ankley and Thomas, 1992 (**57**). 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.

4.3.1.2 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.

4.3.1.3 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 reasonable 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.

4.3.1.4 Natural physico-chemical characteristics such as sediment texture may influence the response of test organisms

(58). 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 section 12.1 and Annex A1 and Annex A2 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 Dewitt et al. 1988, (58), Ankley et al., 1994(59). The use of formulated sediment can also be used to evaluate physico-chemical characteristics of sediment on test organisms Walsh et al., 1991 (60) Suedel and Rodgers, 1994, (61) Kembel et al.,(62) USEPA, 2000,(63), section 7.2 and Guide E1391).

4.3.2 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 10.3.2). A useful summary of field sampling design is presented by Green, 1979 (64). See Section 13 for additional guidance on experimental design and statistics.

4.3.2.1 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 (Table A1.3 in Annex A1 and Table A2.3 in Annex A2) and might include a control sediment or a formulated sediment (section 7.2). 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 (that is, grain size, organic carbon) may be useful in these evaluations. A summary of field sampling design is presented by Green, 1979 (64). See Section 13 for additional guidance on experimental design and statistics.

4.3.2.2 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.

4.3.2.3 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 13). 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.

4.3.2.4 Sediments often exhibit high spatial and temporal variability (65). 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.

4.3.2.5 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 may include compositing cores collected to project depth from a specified dredged material management area.

4.3.2.6 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 E1241). 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 (Guide E729). 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 13).

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; Stephan et al.(66)) are not exceeded, yet organisms in or near sediments are adversely affected Chapman, 1989 (67). 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, whole sediment concentrations have not been strongly correlated to bioavailability Burton, 1991 (**68**). 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 Di Toro et al. 1991(**69**) Giesy et al. 1988 (**70**). 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 Kemp et al. 1988, (**71**). 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 as part of 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 (Test Method E1706, Guide E1525, Guide E1850; Annex A1, Annex A2; USEPA, 2000 (**72**), EPA 1994b, (**73**), Environment Canada 1997a, (**74**), Enviroment Canada 1997b,(**75**)). 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 that 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 USEPA, 2000 (**72**); Sibley et al.1996, (**76**); Sibley et al.1997a, (**77**); Sibley et al.1997b, (**78**); Benoit et al.1997, (**79**); Ingersoll et al.1998, (**80**)). Sublethal endpoints in sediment tests might also prove to be better estimates of responses of benthic communities to contaminants in the field, Kembel et al. 1994 (**81**). Insufficient information is available to determine if the long-term test conducted with *Leptocheirus plumulosus* (Annex A2) is more sensitive than 10-d toxicity tests conducted with this or other species.

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 (Annex A1).

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, 1997, (**82**)) 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 Stemmer et al. 1990b, (**83**), aging (Landrum et al. 1989, (**84**), Word et al. 1987, (**85**), Landrum et al., 1992,(**86**)), 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 Di Toro et al. 1990, (**87**) Di Toro et al. 1991,(**69**). 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 Di Toro et al. 1991, (**69**).

TABLE 2 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 (that is, Apparent Effects Threshold (AET), Effect Range Median (ERM), Probable Effect Level (PEL).

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 2 lists several approaches the USEPA has considered for the assessment of sediment quality USEPA, 1992, (**88**). 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 USEPA, 1989a, 1990a, 1990b and 1992b, (**89, 90, 91, 92** and Wenning and Ingersoll (2002 (**93**)) for a critique of these methods). The sediment assessment approaches listed in Table 2 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, USEPA, 1992c, (**94**). Numeric methods can be used to derive chemical-specific sediment quality guidelines (SQGs). Descriptive methods such as toxicity tests with 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, (Long et al. 1991(**95**) MacDonald et al. 1996 (**96**) Ingersoll et al. 1996 (**97**) Ingersoll et al. 1997 (**98**), Wenning and Ingersoll 2002 (**93**)). 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 (Burton, 1991 (**68**), Chapman 1992, 1997 (**99, 100**).)

5.2 *Regulatory Applications*—Test Method E1706 provides information on the regulatory applications of sediment toxicity tests.

5.3 *Performance-based Criteria:*

5.3.1 The USEPA Environmental Monitoring Management Council (EMMC) recommended the use of performance-based methods in developing standards, (Williams, 1993 (**101**). Performance-based methods were defined by EMMC as a monitoring approach which permits the use of appropriate methods that meet preestablished demonstrated performance standards (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 the USEPA Regional and Headquarters Program offices to discuss the development of standard freshwater, estuarine, and marine sediment testing procedures (USEPA, 1992a, 1994a (**88, 102**)). 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 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, 2000 (**72**) 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 Annex A1 and Annex A2 for a listing of performance criteria for culturing or 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 3; Burton, 1991 (**68**)). 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 difficult and may complicate the interpretation of effects. See USEPA, 2000 (**61**) and Guide E1391. An abundance of the same organism (McGee et al., 1999 (**41**) or organisms taxonomically similar to the test organism in the sediment sample may make interpretation of treatment effects difficult. In addition, the presence of predator may change the outcome of a toxicity test. For example, Redmond and Scott, 1989 (**103**) showed that the polychaete *Nephtys incisa* can consume *Ampelisca abdita* under toxicity test conditions. Similarly, predatory isopods (*Cyathura polita*) have been observed to interfere in 10-d toxicity tests conducted with *Leptocheirus plumulosus* (Peter De Lisle, Coastal Bioanalysts, Gloucester, VA; personal communication).

6.1.2.1 Although disruptive of natural sediment physical features, all test sediments in the *Leptocheirus plumulosus* 28-d sediment test should be press-sieved sometime before testing and re-homogenized immediately before introduction to the test chambers if warranted (section 10.3 and Annex A2). Press-sieving is performed primarily to remove predatory organisms, large debris, organisms used in testing (McGee et al., 1999 (**41**)) or organisms taxonomically similar to the test species. Certain applications may recommend that sediments should not be press-sieved. Also, it may not be necessary to press-sieve sediments if previous experience has demonstrated the absence of potential interferences, including predatory or competitive organisms or large debris, or if large debris or predators can be removed with forceps or other suitable tools. The presence of an abundance of amphipods that are taxonomically similar to the test species should prompt press-sieving. This is particularly true if endemic Ampeliscidae are present and *A. abdita* is the test species because it may be difficult to remove all of the resident amphipods from their tubes. If sediments are sieved, it is desirable to perform select analyses (for example, pore-water metals or DOC, AVS, TOC) on samples before and after sieving to document the influence of sieving on sediment chemistry (USEPA, 1994a (**1**)).

TABLE 3 Advantages and Disadvantages for Use of Sediment Tests (Modified from Swartz (104))

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 standards 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.

6.1.3 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, Karickhoff and Morris, 1985 (**105**)). 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 (Stemmer and Burton, 1990b (**83**)).

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) Davenport and Spacie, 1991 (**106**)). However, lighting typically used to conduct laboratory tests does not include the appropriate spectrum of ultraviolet radiation to photoactivate compounds (Oris and Giesy, 1985 (**107**), Ankley et al. 1994b (**108**)), 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 (Dewitt et al. 1998, (**58**)). 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 (section 12.1 and Annex A1 and Annex A2). The effects of sediment characteristics can also be addressed with regression equations Dewitt et al., 1998 (**58**) Ankley et al., 1994 (**59**). The use of formulated sediment can also be used to evaluate physico-chemical characteristics of sediment on test organisms (Walsh et al., 1991 (**60**), Suedel and Rodgers, 1994 (**63**)).

6.1.7 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. Whole-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, (USEPA, 1992 (**94**)) and certain metals normalized to acid volatile sulfides, (DiToro, 1990, (**87**)). 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, (Lamberson and Swartz, 1998 (**109**)).

6.1.8 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 long-term exposures (Ankley et al., 1994, McNulty et al. 1999 (**59, 110**)). However, the addition of the food may alter the availability of the chemicals in the sediment, (Harkey et al. 1994, Wiederholm et al. 1987 (**111,112**)) depending on the amount of food added, its composition (for example, total organic carbon (TOC)), and the chemical(s) of interest.

6.1.9 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 (Ankley and Thomas, 1992, (**57**)). Laboratory studies that test single compounds spiked into the sediment can be used to determine more directly the specific chemicals causing a toxic response (Swartz et al. 1998 (**113**)).

6.1.10 Sediment spiking can also be used to investigate additive, antagonistic, or synergistic effects of specific chemical mixtures in a sediment sample (Swartz et al, 1998 (**113**)). However, spiked sediment may not be representative of contaminated sediment in the field. Mixing time (Stemmer et al. 1990a (**65**)), and aging (Landrum 1999, Word et al. 1997, Landrum and Faust 1992 (**84, 85, 86**) of spiked sediment can affect responses of organisms.

6.1.11 Salinity of the overlying water is an additional factor that can affect the bioavailability of metals. Importantly, some metals (for example, cadmium) are more bioavailable at lower salinities. Therefore, if a sediment sample from a low salinity location is tested with overlying waters of high salinity, there is the potential that metal toxicity may be reduced. The suite of species provided in this standard allow these tests to be conducted over the range of pore-water salinities routinely encountered in field-collected sediments from North American estuarine or marine environments (USEPA 1994a (**1**)). In addition, artificial sea salts may contain chelating agents (EDTA) that can potentially influence the bioavailability of metals. Certain brands of artificial salts are available from manufacturers without the addition of sodium thiosulfate that can also influence the toxicity of contaminants.

6.1.12 Most assessments of contaminated sediment rely on acute-lethality testing methods (for example, <10 d; (USEPA-USACE 1977, 1991, 1998, (**114, 115, 116**)). 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 (Dillon et al. 1994, Kemble et al. 1994, Ingersoll and Brunson 1998, (**117, 81, 80**), Annex A2). Insufficient information is available to determine if the long-term test conducted with *Leptocheirus plumulosus* (Annex A2) is more sensitive than 10-d toxicity tests conducted with this or other species.

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 (for example, Canfield et al.. (**118**)). 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, USEPA 1986a, Swartz 1989, (**119, 104**)). 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 Annex A1 and Annex A2.

7. Reagents and Materials

7.1 *Water:*

7.1.1 *Requirements*—Sea water used to test and culture organisms should be uniform in quality. Acceptable sea 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 (1993 (**120**)) and Guide E729 for a recommended list of chemical analyses of the water supply.

7.1.2 *Source:*

7.1.2.1 Culture and testing water can be natural or synthetic seawater (USEPA-USACE 2001 (**2**)).

7.1.2.2 The source of natural water will depend to some extent on the objective of the test and the test organism that is being used. All natural waters should be obtained from an uncontaminated surface-water source beyond the influence of known discharges. It may be desirable to collect water at slack high tide, or within one h after high tide. Suitable surface water sources should have intakes that are positioned to: *(1)* minimize fluctuations in quality and contamination, *(2)* maximize the concentration of dissolved oxygen (DO), and *(3)* ensure low concentrations of sulfide and iron. For estuarine tests, water having a salinity as near as possible to the desired test salinity should be collected from an uncontaminated area.

7.1.2.3 Alternatively, it may be desirable to dilute full strength sea water with an appropriate fresh water source. Sources of fresh water (that is, 0 ‰) for dilution include deionized water, uncontaminated well or spring water, or an uncontaminated surface-water source. 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 to dilute water utilized 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 for diluting sea water to the desired salinity since dechlorination is often incomplete (Guide E729; USEPA, 1993 (**120**)). It might be desirable or necessary to dilute full strength seawater with an appropriate freshwater source to achieve 5 % or 20 % (or the selected salinity; section 1.4) used in culturing or testing of *L. plumulosus* (USEPA-USACE 2001 (**2**), Section 12.

7.1.2.4 For site-specific investigations, it may be desirable to have the water-quality characteristics of the overlying water (that is, salinity) as similar as possible to the site water (section 1.4). For certain applications the experimental design might require use of water from the site where sediment is collected. In estuarine systems, however, the pore-water salinity of sediments may not be the same as the overlying water at the time of collection (Sanders et al., 1965 (**121**)).

7.1.2.5 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 or less.

7.1.2.6 Natural sea water might need aeration using air stones, surface aerators, or column aerators. Adequate aeration will stabilize pH, bring concentrations of DO and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. The concentration of DO in source water should be between 90 to 100 % saturation to help ensure that DO concentrations are acceptable in test chambers. Natural sea water used for holding or acclimating, culturing, and testing amphipods should be filtered (<5 µm) shortly before use to remove suspended particles and organisms.

7.1.2.7 Water that is prepared from natural sea water should be stored in clean, covered containers at 4°C. USEPA-USACE (2001(**2**)) states that natural sea water should be used within 2 d for larval toxicity tests (Woelke, 1968 (**122**),1972 (**123**) ; Cardwell et al., 1977 (**124**),1979 (**125**)). However, investigators have found that when sea water is continuously aerated, it can be held for up to a month before use with certain species (David Moore, MEC Analytical, Carlsbad, CA; personal communication).

7.1.3 *Reconstituted/Synthetic Seawater:*

7.1.3.1 Although reconstituted water is acceptable, natural seawater is preferable, especially for tests involving chemicals whose bioavailability is affected by seawater chemistry. Reconstituted water can be prepared by adding specified amounts of reagent- grade chemicals to high-purity deionized water (Guide E729; USEPA, 1993 (**120**)). Acceptable high-purity water can be prepared using deionization or reverse-osmosis units (section 7.1; USEPA, 1993 (**120**)). Test water can also be prepared by diluting natural water with deionized water (Kemble et al., 1994 (**50**)).

7.1.3.2 Deionized water should be obtained from a system capable of producing at least 1 MΩ (mega-ohms) 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.

7.1.3.3 Reconstituted sea water is prepared by adding specified amounts of a suitable salt reagent to high-purity deionized water (Guide E729, USEPA, 1991(**126**)). Suitable salt reagents can be reagent grade chemicals, or commercial sea salts. Pre-formulated brine (for example, 60 to 90 %), prepared with dry ocean salts or heat-concentrated natural sea water, can also be used. (USEPA, 1994 (**1**) USEPA -USACE 2001, (**2**))

7.1.3.4 A synthetic sea formulation called GP2 is prepared with reagent grade chemicals that can be diluted with a suitable high-quality water to the desired salinity (USEPA, 1994b (**127**)).

7.1.3.5 The suitability and consistency of a particular salt formulation for use in holding and testing should be verified by laboratory tests because some formulations can produce unwanted toxic effects or sequester contaminants (Environment Canada, 1992 (**5**) ; USEPA-USACE 2001(**2**)). In controlled tests with the salt formulations mentioned above, Emery et al.

(1997 (**8**)) found differences in survival, growth, and reproduction, and that laboratories can have acceptable performance (that is, survival) with any of the salts evaluated. Because of higher growth rates observed in the Crystal Sea Marinemix® seasalt, they recommended its use for culturing and testing of *L. plumulosus* (Emery et al., 1997 (**8**); Annex A2).

7.1.3.6 To obtain the desired holding or acclimation salinity, sea salts or a hypersaline solution (USEPA, 1993 (**120**)) brine can be added to a suitable freshwater, deionized water, estuarine water, or the laboratory's sea water supply may be diluted with a suitable freshwater or deionized water.

7.1.3.7 Salinity, pH, and DO should be measured on each batch of reconstituted water. The reconstituted water should be aerated before use to adjust pH and DO to the acceptable ranges (for example, section 7.1). The artificial sea salts should be held for at least two week before use to allow pH to become more stable and reduce the activity of chelating agents (Environment Canada 1992 (**5**)).

7.2 *Formulated Sediment*—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 USEPA 2000, (**61**), Test Method E1706 and Guide E1391 for additional detail regarding preparation and use of formulated sediment.

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.

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 USEPA 1986b, Walters and Jameson 1984, (**128, 129**).

8.6 *Pollution Prevention, Waste Management, and Sample Disposal*—Guidelines for the handling and disposal of hazardous materials should be strictly followed (Guide D4447). 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., 1986 (**130**)) 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 (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 Table A1.3 in Annex A1 and Table A2.3 in Annex A2). Evaluations may also be made on the magnitude of the within-chamber and between-chamber variance in a test (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 lux 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 ($\mu mol^{-2}\ s^{-1} = W\ m \times \lambda(nm) \times 8.36 \times 10^{-3}$)). 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. A16:8 light:dark photoperiod should be used for culturing *L. plumulosus* (section 12.6) and for holding and acclimating *A. abdita* in the laboratory before testing (12.4; USEPA 1994a (**1**)).

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 (USEPA 2000, (**72**)). 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.

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 (15 to 25°C).

9.3.3.2 *Water Purification System,* capable of producing at least 1 MΩ (mega-ohms) of water (USEPA, 1993a (**131**)).

9.3.3.3 *Analytical Balance,* capable of accurately weighing to 0.01 mg (for the *L. plumulosus* test).

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 (for the *L. plumulosus* test).

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 Pipettes,* Class A, 1 to 100 mL.

9.3.3.7 *Serological Pipettes,* 1 to 10 mL, graduated.

9.3.3.8 *Pipette 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, 1997b, (**132**)).

9.3.3.13 *Dissolved Oxygen (DO), pH/Selective Ion, and Specific Conductivity Meters and Probes and temperature-compensated refractometer,* 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 Equipment for measuring ammonia (that is, an ammonia-specific probe or an ammonia test kit) is also necessary.

9.3.3.15 See USEPA (1994a(**1**)) and USEPA-USACE (2001 (**2**)) for a list of additional equipment and supplies.

9.3.4 *Test Chambers*—Test chambers to be used in sediment toxicity tests are 1-L glass containers (beakers or wide-mouth jars) with an internal diameter of about 10 cm. Each test chamber should have a cover. Acceptable covers include watch glasses, plastic lids, glass culture dishes, or parafilm. It may be necessary to drill a hole in the glass cover to allow the insertion of a pipette for aeration (USEPA 1994a (**1**)).

9.3.5 *Cleaning:*

9.3.5.1 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.

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

9.3.5.3 Rinse twice with tap water.

9.3.5.4 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.

9.3.5.5 Rinse twice with deionized water.

9.3.5.6 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.

9.3.5.7 Rinse three times with deionized water.

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

9.3.5.9 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 (Guide E729), 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 (Stemmer et al. 1990a (**65**)). 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 for some experimental designs. Sampling may cause loss of sediment integrity, change in chemical speciation, or disruption of chemical equilibrium (Guide E1391). 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.

10.1.3 Exposure to direct sunlight during collection should be minimized, especially if the sediment contains photolytic compounds (Davenport and Spacie 1991, Oris and Giesy 1985, (**106, 107**)). Sediment samples should be cooled to 4°C in the field before shipment (Guide E1391). 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 (USEPA 2000, (**72**)).

10.1.4 For additional information on sediment collection and shipment see Test Method E1706, Guide E1391, USEPA, 2000 (**61**), and USEPA, 2000 (**133**) 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 E1391) to less than eight weeks (USEPA-USACE, 1998) (**134**). 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. 1994, (**135**); Becker et al. 1995, (**136**), Carr and Chapman 1995, (**137**), Moore et al. 1996, (**138**), Sarda and Burton 1995, (**139**), Sijm et al. 1997, (**140**), DeFoe and Ankley 1998, (**141**)). 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 1995 (**139**)). 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 1995 (**137**); Moore et al. 1996 (**138**); DeFoe and Ankley 1998 (**141**)). 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 1998 (**141**) observed high variability in survival during early testing periods (for example, <2 weeks) in sediments with low toxicity. De Foe and Ankley 1998, (**141**) 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 (for example, 2 weeks) to reduce potential for interferences from indigenous organisms. Sediments that contain comparatively stable compounds (for example, 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. 1996 (**138**), DeFoe and Ankley 1998, (**141**)). For these sediments, long-term storage (for example, >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 (Kemble et al. 1994, (**81**)). Ingersoll et al. 1993, (**142**) 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 E1391; Schuytema et al. 1989, (**143**)). 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. 1978,(**144**)). 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., 1994 (**145**), 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 (Guide E1391). 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 (Kemble et al. 1994, (**81**)).

10.3.2 *Sediment Spiking:*

10.3.2.1 Test sediment can be prepared by manipulating the properties of a control sediment. Mixing time (Stemmer et al. 1990a, (**65**)) and aging (Landrum 1989, Word et al. 1987, Landrum and Faust 1992, (**84, 85, 86**)) 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 chemicals (section 10.3.2.6). 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 2000, (**61**) and Guide E1391 for additional detail regarding sediment-spiking techniques.

10.3.2.2 The cause of sediment toxicity and the interactive effects of chemicals can be determined by spiking a sediment with chemicals or complex waste mixtures (Lamberson and Swartz 1988, (**109**)). 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; (Ankley et al. 1992b, **(146)**). The influence of sediment physico-chemical characteristics on chemical toxicity can also be determined with sediment-spiking studies Swartz et al. 1994, **(147)**.

10.3.2.3 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 E1391; 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 E1391, USEPA 2000, **(133)**). Duration of contact between the chemical and sediment can affect partitioning and bioavailability Word et al. 1987, **(85)**. 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 Ditsworth, et al. 1990 **(148)**. Moreover, results from sediment-spiking studies should be compared with the response of test organisms to chemical concentrations in natural sediments (Lamberson and Swartz 1992 **(149)**).

10.3.2.4 Organic chemicals have been added: *(1)* directly in a dry (crystalline) form; *(2)* coated on the inside walls of the container (Ditsworth et al.1990, **(148)**); or *(3)* coated onto silica sand (for example, 5 % w/w of sediment) which is added to the sediment (Test Method E1706). 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.

10.3.2.5 Metals are generally added in an aqueous solution (Guide E1391; Di Toro et al.1990, **(87)**). Ammonia has also been successfully spiked using aqueous solutions (Besser et al. 1998, **(150)**). Inclusion of spiking blanks is recommended.

10.3.2.6 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 (for example, >6; Test Method E1706). 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.

10.3.2.7 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 E1391). 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.

10.3.2.8 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 E1241). 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:*

10.3.3.1 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 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 (Di Toro et al. 1991, **(69)**). See Section 13 for a description of procedures to analyze data generated from these studies.

10.3.3.2 Whole-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 (DiToro 1990, 1991, **(87, 69)**.

10.3.3.3 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: salinity, 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 (Plumb, 1981, **(151)**). See section 10.4.5 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, dissolved organic carbon, organic nitrogen, metals, synthetic organic compounds, oil and grease, and petroleum hydrocarbons in sediment and interstitial water.

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.

10.4.5 *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 benchmarks (ESBs) 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. 1991, **(69)**). Of additional importance is contaminants in interstitial waters can be transported into overlying waters through diffusion, bioturbation, and resuspension processes (Van Rees et al. 1995, **(152)**). 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.

10.4.5.1 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 1995, **(153)**, Guide E1391, and USEPA 2000, **(61)**.

10.4.5.2 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, 1995 **(154)**), squeezing (Carr and Chapman, 1995 **(137)**) and suction (Winger and Lasier, 1998 **(155)** ; Winger et al. **(154)**), 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, 1992, **(156)** ; Sarda and Burton, 1995, **(139)**).

10.4.5.3 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 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, 1995 **(157, 153)**; Winger et al. 1998, **(155)**), which vary with sediment characteristics, chemicals of concern, toxicity test methods, and desired test resolution (that is, 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. 1994, **(59)**; Horowitz et al. 1992, **(158)**). For most toxicity test procedures, relatively large volumes of interstitial water (for example, 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. 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 2001 (**133**) and Guide E1391 for additional detail regarding isolation of interstitial water.

10.4.5.4 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 (that is, 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. 1994, (**59**). For most toxicity test procedures, relatively large volumes of interstitial water (for example, 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. 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 2001, (**133**) and Guide E1391 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).

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 USEPA 1993a, 1993c, 1995, 1978, 1979a, 1980a, 1980b, 1993b, and DeWoskin 1984, (**131, 159, 160, 161, 162, 163, 164, 165, 166**).

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 (Williams 1993, (**101**)). 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 *L. plumulosus*, it was determined that no single method has to be used to culture organisms (USEPA-USACE 2001 (**2**)). 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 (that is, size) for testing is the key consideration relative to culture methods. Therefore, a performance-based criteria approach is the preferred method by which individual laboratories should evaluate culture health, rather than a control-based criteria approach. Performance-based criteria were chosen to allow each laboratory to optimize culture methods that provide organisms that produce reliable and comparable test results. Performance criteria for culturing and testing *L. plumulosus* are listed in Table A2.3 of Annex A2.

11.3 *Facilities, Equipment, and Test Chambers:*

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 (section 11.14).

TABLE 4 Recommended Test Conditions for Conducting Reference-Toxicity Tests (USEPA 1994a (1))

Parameter	Conditions
1. Test Type:	Water-only test
2. Dilution series:	Control and at least 5 test concentrations (0.5 dilution factor)
3. Toxicant:	Cd, Cu, ammonia, Sodium dodecyl sulfate (SDS)
4. Temperature:	15°C *E. estuarius* and *R. abronius* 20°C *A. abdita* 25°C *L. plumulosus*
5. Light quality:	Chambers should be kept in the dark covered with opaque material (USEPA 1994a **(1)**). Alternatively USEPA-USACE (2001**(2)**) recommends a 500 to 1000 lux light intensity at a 16:8 light:dark cycle for *L. plumulosus* in long-term tests (Annex A2).
6. Photoperiod:	24 h dark
7. Salinity	28 ‰ for *A. abdita* and *R. abronius* 20 ‰ for *E. estuarius* 5 or 20 ‰ for *L. plumulosus.* Alternatively, the salinity of the overlying water can be adjusted to the salinity of the pore-water at the site of interest in tests with *E. estuarius* or *L. plumulosus*. If tests are conducted at different salinities, additional tests are required to determine comparability of results (sections 1.4 and 1.10).
8. Renewal of water:	None
9. Age of organisms:	*A. abdita:* 3 to 5 mm (no mature males or females) *E. estuarius* and *R. abronius:* 3 to 5 mm *L. plumulosus:* 2 to 4 mm (no mature males or females; USEPA 1994a **(1)**). Alternatively, USEPA-USACE (2001 **(2)**) recommend testing *L. plumulosus* in a range of 0.25 to 0.60 mm in length in long-term tests (Annex A2).
10. Test chamber:	250-ml to 1-L glass beaker or jar
11. Volume of water:	80 % of chamber volume
12. Number of organisms/ chamber:	n = 20 if 1 replicate; n = 10 (minimum) if >1 per replicate.
13. Number of replicate chambers/treatment:	1 minimum; 2 recommended
14. Aeration:	Recommended; but not necessary if >90 % dissolved oxygen saturation can be achieved without aeration (USEPA 1994a **(1)**). Alternatively USEPA-USACE (2001 **(2)**) recommends that dissolved oxygen should be maintained at >60 % saturation (>4.4 mg/L).
15. Dilution water:	Culture water, surface water, site water, or reconstituted water
16. Water quality:	Salinity, pH, and dissolved oxygen, at the beginning and end of a test. Temperature daily.
17. Test duration:	96 h
18. Endpoint:	Survival (LC50); Reburial (EC50) optional for *E. estuarius* and *R. abronius*
19. Test acceptability:	90 % control survival

11.4 *Test Organisms*—The organisms should appear healthy, behave normally, feed well, and have low mortality in cultures of *L. plumulosus*, 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 (section 12.3.3.9).

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 7.1 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, salinity, conductivity (particularly when salinity is <1 ‰), ammonia, and pH should be checked in accordance Annex A1 and Annex A2.

11.8 *Quality of Test Organisms:*

11.8.1 If test organisms are obtained from culture, reference-toxicity tests should be conducted on all test organisms using procedures outlined in section 11.16 (at a minimum, one test every six months; Table 4). If reference-toxicity tests are not conducted monthly, the lot of organisms used to start a sediment test should be evaluated using a reference toxicant (USEPA 1994a (**1**)).

11.8.2 The quality of test organisms obtained from an outside source, regardless of whether they are from culture or collected from the field, should be verified by conducting a reference-toxicity test concurrently with the sediment test (USEPA 1994a (**1**)). For cultured organisms, the supplier should provide data with the shipment describing the history of the sensitivity of organisms from the same source culture. For field-collected organisms, the supplier should provide data with the shipment describing the collection location, the time and date of collection, the water salinity and temperature at the time of collection, and collection site sediment for holding and acclimation purposes. If the supplier has not conducted reference toxicity tests with the test organism, it is the responsibility of the testing laboratory to conduct these reference toxicity tests (section 11.14.1).

11.8.3 *Leptocheirus plumulosus* for chronic testing can be obtained from laboratory cultures (USEPA-USACE 2001 (**2**)) or from commercial sources. It is likely to be impractical to obtain test-size neonates directly from a supplier because of their sensitivity to physical disturbances and their rapid

growth. Instead, test laboratories will likely want to establish their own cultures of *L. plumulosus* from which to harvest neonates (section 12.5). It is desirable to determine the sensitivity of *L. plumulosus* obtained from an outside source. For cultured organisms, the supplier should provide data with the shipment describing the history of the sensitivity of organisms from the same source culture. For field-collected organisms, the supplier should provide data with the shipment describing the collection location, the time and date of collection, the water salinity and temperature at the time of collection, and collection site sediment for holding and acclimation purposes. The supplier should also certify the species identification of the test organisms and provide the taxonomic references (for example, Shoemaker, 1932 (**167**); Bousfield, 1973 (**168**)) or name(s) of the taxonomic expert(s) consulted.

11.8.4 All organisms in a test must be from the same source (Section 12). Organisms may be obtained from laboratory cultures or from commercial or government sources (section 11.8.3). The test organisms used should be identified using an appropriate taxonomic key, and verification should be documented. The use of field-collected *L. plumulosus* to start cultures is discussed in section 12.3. Obtaining organisms from wild populations is useful for enhancement of genetic diversity of existing cultures or to establish new cultures. (McGee et al. 1998 (**169**)) found seasonal variability in sensitivity to cadmium in field-collected *L. plumulosus*. Therefore, field-collected *L. plumulosus* should not be used for toxicity testing 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., 1997 (**170**)). Sensitivity of the wild population to select contaminants 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 *L. plumulosus* in cultures (section 12.5). Additionally, survival in sediment tests conducted with *A. abdita* and *L. plumulosus* may be affected by the nutritional suitability of food provided during holding and acclimation (USEPA 1994a (**1**)).

11.10 *Test Acceptability*—Table A1.3 in Annex A1 and Table A2.3 in Annex A2 outline requirements for acceptability of tests. An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests. 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 (Van Rees et al. 1991, Bufflap and Allen 1995, Ankley and Subauer-Bergian 1995, 152-154).

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, DO, temperature, salinity, 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, salinity, conductivity (particularly when salinity is <1 ‰)). 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 (Annex A1 and Annex A2).

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 (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 (that is, water quality) result in acceptable performance in the tests as outlined in Annex A1 and Annex A2.

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 Tomasovic et al. 1995, (**171**).

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 (section 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 Annex A1 and Annex A2 must 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 (Section 15).

11.16 *Reference-toxicity Testing:*

11.16.1 Reference-toxicity tests should be conducted in conjunction with sediment tests to determine possible changes in condition of a test organism (Lee, 1980 (**172**)). Water-only reference-toxicity tests on cultured organisms should be conducted least every 6 months with laboratory-cultured organisms and should be performed on each batch of field-collected organisms used for testing. Deviations outside an established normal range may indicate a change in the condition of the test organism population. Results of reference-toxicity tests also enable interlaboratory comparisons of test organism sensitivity (USEPA 1994a (**1**)).

11.16.2 Reference toxicants such as cadmium (available as cadmium chloride ($CdCl_2$), copper (available as copper sulfate ($CuSO_4$), ammonia, and sodium dodecyl sulfide (SDS) are suitable for use. 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 (Lee, 1980 (**172**)). However, it may be unrealistic to test more than one or two reference toxicants routinely.

11.16.3 Test conditions for conducting reference-toxicity tests with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* are outlined in Table 4.

11.16.4 Based on 96-h, water-only reference-toxicity tests at 20 ‰ with neonate *L. plumulosus*, one should expect a mean LC50 value for cadmium of about 0.5 mg/L (range: 0.2 mg/L to 0.7 mg/L) and LC50 values for total ammonia between 25 mg/L and 60 mg/L (DeWitt et al., 1997a(**7**)). At 5 ‰, one should expect a mean LC50 value for cadmium of about 0.05 mg/L (range: 0.01 mg/L to 0.09 mg/L) and LC50 values for total ammonia between 37 mg/L and 53 mg/L (Emery et al., 1997 (**8**); Moore et al., 1997 (**27**)). Kohn et al. 1994 (**25**) report 96-h LC50 values for total ammonia (mg/L) of 79 for *R. abronius*, 126 (estimated) for *E. estuarius*, and 50 for *A. abdita*. Northwestern Aquatic Sciences (NAS) reports an average total ammonia (mg/L) 96-h LC50 of 138 for *E. estuarius* (n=5), and mean (n=20) 96-h LC50 values for cadmium of 1.1 for *R. abronius*, 2.4 for *E. estuarius*, and 0.55 for *A. abdita*. The NAS laboratory also reports a mean (n=20) sodium dodecyl sulfate (SDS; mg/L) 48-h LC50 of 12.6 for *A. abdita*. All of the NAS tests were conducted at 28 ‰ except for the *E. estuarius* cadmium test, which was conducted at 12 ‰ (Michele Redmond, Northwestern Aquatic Sciences, Newport, OR, personal communication). MEC Analytical has observed 96-h LC50 values for cadmium (mg/L) of 0.75 for *R. abronius*, 7.1 for *E. estuarius*, 0.56 for *A. abdita*, and 3.9 for *L. plumulosus* (David Moore, MEC Analytical, Carlsbad, CA, personal communication). DeWitt et al. (1992a (**6**)) provides additional data on cadmium reference toxicity tests for *R. abronius*, *E. estuarius*, *A. abdita*, and *L. plumulosus*.

11.17 *Record Keeping*—Section 13.1 outlines recommendations for recorded keeping (that is, data files, chain-of custody).

12. Collection, Culturing, and Maintaining Test Organisms

12.1 *Life History:*

12.1.1 *Ampelisca abdita*— *A. abdita* is a tube-building amphipod in the family Ampeliscidae. It occurs on the Atlantic coast from central Maine to central Florida, although it is also found in the eastern portion of the Gulf of Mexico (Bousfield, 1973 (**168**)). On the Pacific coast, it is present in San Francisco Bay, CA (Nichols et al. 1985 (**173**); Hopkins, 1986 (**174**)). They are small (adult length 4 to 8 mm), laterally compressed amphipods. Healthy animals are opalescent pink and will remain tightly curled, whereas unhealthy animals tend to be translucent white, and may uncurl (USEPA 1994a (**1**)). Often dominant members of the benthic community, *A. abdita* forms thick mats of tubes with amphipod densities up to 110,000/m^2, and are often a dominant food source for bottom-feeding fish (Richards, 1963 (**175**)). The tubes are narrow and about 2 to 3 cm in length. A filter feeder, *A. abdita* feeds on both particles in suspension and those from surficial sediment surrounding the tube. *Ampelisca abdita* is euryhaline, and has been reported in waters that range in salinity from fully marine to 10 ‰ (Hyland, 1981 (**176**)). Laboratory tests have shown the salinity application range of *A. abdita* in sediments is from 0 to 34 ‰ when the salinity of overlying water is 28 ‰ (Weisberg et al. 1992, (**177**)). This species generally inhabits sediments from fine sand to mud and silt without shell fragments, although it can also be found in relatively coarser sediments with a sizeable fine component. It is often abundant in sediments with a high organic content. Analysis of historical data shows little effect of sediment grain size on survival of *A. abdita* during 10-dsediment toxicity tests (Long and Buchman, 1989 (**18**) ; Weisberg et al. 1992, (**177**)). There is evidence that sediments with >95 % sand may elicit excessive mortality (John Scott, personal communication in USEPA 1994a (**1**)). *Ampelisca abdita* have been collected at water temperatures ranging from -2 to 27°C (USEPA 1994a (**1**)). Reproduction patterns of *A. abdita* vary geographically. In the colder waters of its range, *A. abdita* produces two generations per year, an over-wintering population that broods in the spring, and a second that breeds in mid- to late-summer (Mills, 1967 (**178**)). In warmer waters south of Cape Hatteras, NC, breeding might be continuous

throughout the year (Nelson, 1980 (**179**)). Juveniles are released after about two weeks in the brood pouch. Juveniles take about 40 to 80 d to become breeding adults at 20°C (Mills, 1967 (**178**); Scott and Redmond, 1989 (**12**); Redmond et al. 1994 (**180**)).

12.1.2 *Eohaustorius estuarius*— *E. estuarius* is a free-burrowing amphipod in the family Haustoriidae. It is found on protected and semi-protected beaches from the lower intertidal to shallow subtidal waters exclusively on the Pacific coast from British Columbia south to central California (Environment Canada, 1992 (**5**); USEPA-USACE 2001 (**2**)). They are stout (adult size range 3 to >5 mm) cup- or bell-shaped, dorsally compressed amphipods that are grayish-brown or yellowish-brown in color (Environment Canada, 1992 (**5**)). *Eohaustorius estuarius* are thought to be deposit feeders. It is an estuarine species and has been reported in areas where pore-water salinity ranges from 1 to 35 % (Environment Canada, 1992 (**5**), USEPA 1994a (**1**) ; Michele Redmond, Northwest Aquatic Sciences, Newport, OR; personal communication). Laboratory studies have shown a salinity application range in control sediments for *E. estuarius* from 0 to 34 ‰ . *Eohaustorius estuarius* inhabits clean, medium-fine sand with some organic content. The species has exhibited acceptable (that is, >90 %) survival when exposed to clean sediments with a wide range of grain sizes, with generally little affect on survival whether coarse-grained or fine-grained (that is, predominantly silt and clay) clean sediments are used (Environment Canada, 1992 (**5**)). Environment Canada (1998 (**181**)) reported that *E. estuarius* can tolerate up to 70 % clay in sediment toxicity tests. However, some correlation between survival and grain size exists (DeWitt et al., 1989 (**11**)). *Eohaustorius estuarius* has been collected from water temperatures from 0 to 23°C (USEPA-USACE 2001 (**2**)). *Eohaustorius estuarius* apparently has an annual life cycle (Environment Canada, 1992 (**5**) ; DeWitt et al. 1989 (**11**)). Gravid females are abundant in intertidal sediments from February through July. However, reproduction might occur year-round because juveniles are found throughout most of the year (DeWitt et al., 1989 (**11**)).

12.1.3 *Leptocheirus plumulosus*— *L. plumulosus* is a burrow-building member of the family Aoridae. It is an infaunal amphipod found in subtidal portions of Atlantic Coast brackish estuaries from Cape Cod, Massachusetts, to northern Florida (Bousfield, 1973 (**168**); DeWitt et al., 1992a (**6**)). It is common in protected embayments, but has been collected in channels of estuarine rivers at water depths up to 13 m (Shoemaker, 1932 (**167**); Holland et al., 1988 (**182**); Schlekat et al., 1992 (**13**)). In Chesapeake Bay, densities of *L. plumulosus* can reach 40,000/m^2 (McGee 1998, (**183**)). *L. plumulosus* is a relatively large amphipod (adult length up to 13 mm) with a cylindrically shaped body that is brownish-gray in color. A distinguishing feature is a series of dark bands or stripes that cross the dorsal surface of the pareons and pleons. It feeds on particles that are in suspension and on the sediment surface (DeWitt et al., 1992a (**6**)). Studies have shown that *L. plumulosus* population abundance in Chesapeake Bay is negatively correlated with sediment contamination (Holland et al., 1988 (**182**); McGee and Fisher 1999 (**42**); McGee et al. 1999 (**41**)). Thus, this amphipod would appear to be a good candidate to be an environmental indicator. *L. plumulosus* is found in both oligohaline and mesohaline regions of east coast estuaries; ambient water salinity at collection sites has ranged from 0 to 15 ‰ (Holland et al., 1988 (**182**); DeWitt et al., 1992a (**6**); Schlekat et al., 1992 (**13**), 1994(**184**)). Laboratory studies have demonstrated that *L. plumulosus* 28-d test can be conducted at salinity values ranging from 1 to 35 ‰ (A2.4; Schlekat et al., 1992 (**13**); DeWitt et al., 1992a (**6**), 1997a (**7**); Emery et al., 1997 (**8**)). This amphipod is most often found in fine-grained sediment with a relatively high proportion of particulate organic material, although it has been collected in fine sand with low organic content (Jordan and Sutton, 1984(**185**); Holland et al., 1988 (**182**); Marsh and Tenor, 1990(**186**); DeWitt et al., 1992a (**6**); Schlekat et al., 1992 (**13**); 1994 (**184**)). Laboratory studies with *L. plumulosus* revealed no effect of sediment grain size on survival in control sediment containing 5 to 100 % silt-clay content (DeWitt et al., 1997a (**7**)). However, Emery et al. (1997 (**8**)) found significantly reduced survival in sediments in which clay content exceeded 84 %. Populations of *L. plumulosus* can be seasonally ephemeral with major population growth in fall and spring and large population declines in the summer (Holland et al., 1988 (**182**); Marsh and Tenore, 1990 (**186**); McGee, 1998 (**183**)). This pattern appears to be driven by changes in temperature and food availability and subsequent effects on life history traits (Marsh and Tenore, 1990 (**186**); McGee, 1998 (**183**)). Short-term population fluctuations are also a function of its relatively short generation time (DeWitt et al., 1992a (**6**)). At 28°C in the laboratory, the age of the first brood release is about 24 d (DeWitt et al., 1992a (**6**)).

12.1.4 *Rhepoxynius abronius* is a free-burrowing amphipod in the family Phoxocephalidae. It occurs on the Pacific Coast from Puget Sound, WA, to central California in lower intertidal and nearshore subtidal zones to depths of 274 m offshore (Environment Canada, 1992 (**5**); Lamberson and Swartz, 1988 (**187**); Kemp et al. 1985 (**188**); Barnard and Barnard, 1982 (**189**)). Densities in the field are reported to range from 150 to 2200/m^2 (Lamberson and Swartz, 1988 (**187**); Swartz et al. 1985 (**10**)). It is a medium-sized (adult length from 3 to >5 mm) amphipod with a stout, somewhat rounded body shape. Color may range from salmon pink to yellowish, grayish-brown to white with a pinkish- brown hue (Environment Canada, 1992 (**5**)). *Rhepoxynius abronius* is a meiofaunal predator, but it also ingests sedimentary organic material (Oakden 1984 (**190**)). In the field, *R. abronius* is found where pore-water salinity is no lower than 20 ‰ (Environment Canada, 1992 (**5**)). Laboratory tests have indicated that salinities below 25 ‰ may be toxic to *R. abronius* (Swartz et al., 1985 (**10**)). *Rhepoxynius abronius* should therefore normally not be chosen as the test species when the sediment pore water is <25 % (Swartz et al., 1985 (**10**)). PSEP (1995 (**191**)) outlines a procedure for adjusting the pore-water salinity of samples with a salinity <25 % by adding appropriately saline overlying water to the test chamber on the day before the start of the test, mixing sediment and overlying water, and allowing the material to settle overnight under aeration. The resultant overlying water is either retained or about 75 % replaced with fresh

TABLE 5 Comparison of Habitat Characteristics and Other Life History Parameters of four Estuarine or marine Amphipod Species Used in Sediment Toxicity Tests (USEPA 1994a (1))

Criterion	*Ampelisca abdita*	*Eohaustorius estuarius*	*Leptocheirus plumulosus*	*Rhepoxynius abronius*
Substrate Relation	Tube dwelling, closed and well developed[A]	Free burrowing[B]	Tube dwelling, open and less developed[A]	Free burrowing[C]
Zoogeography	Atlantic-Gulf[A] San Francisco[D,E]	Pacific[B,F]	Atlantic[A]	Pacific[C]
Habitat	Poly-upper mesohaline[A]	Oligo-mesohaline[B,F]	Oligo-mesohaline[A]	Polyhaline[C,G]
Life cycle	40 to 80 days[H]	Annual[B]	30 to 40 days[I, J,K]	Annual[L]
Availability	Field or potential laboratory culture[A]	Field[B]	Field and laboratory culture[I,J,K]	Field[G]
Ecological importance	High	High	High[I]	High

[A] Bousfield, 1973 (168)
[B] DeWitt et al., 1989 (11)
[C] Barnard and Barnard, 1982 (189)
[D] Nichols et al., 1985 (173)
[E] Hopkins, 1986 (174)
[F] Environment Canada, 1992 (5)
[G] Swartz et al., 1985 (10)
[H] Scott and Redmond, 1989 (12)
[I] DeWitt et al., 1992a (6)
[J] Schlekat et al., 1992 (13)
[K] McGee et al., 1993 (20)
[L] Kemp et al., 1985 (188)

dilution water at 28 ‰ (PSEP 1995 (191)). While this manipulation should result in an acceptable salinity for tests with *R. abronius*, the influence of this manipulation on the bioavailability of contaminants in the sediment sample is uncertain. *Rhepoxynius abronius* naturally inhabits clean, fine, sandy sediments. A number of studies have shown some reduction in survival when this species is held in very fine-grained (predominantly silt and clay) sediment (DeWitt et al., 1988 (192); Long et al., 1990 (29); McLeay et al., 1991 (193)). Normally collected at temperatures ranging from 8 to 16°C, *R. abronius* has survived at temperatures ranging from 0 to 20°C under laboratory conditions. Reproduction of *R. abronius* is annual, with peak production occurring from late winter through spring (Kemp et al., 1985 (188)).

12.2 *Species Selection*—All four species have been routinely used to test sediments with a range of grain size characteristics and pore-water salinities. Selection of one or more of the four species for a particular test/investigation should take into consideration the geographic location of the testing facility and study area, the pore-water salinity regime of the study area, and the grain size characteristics of the sediment being tested. The species that is used should exhibit tolerance to the physicochemical properties of every sediment included in a particular study. Pore-water ammonia concentrations may also enter into selection of one species over others because the four species exhibit differential sensitivity to aqueous ammonia. Most often it will not be necessary to discriminate among the four species, and the decision to test one species above the rest may be driven by practical or logistical concerns. For example, a testing facility may choose to primarily test one species with a suitable local population in order to prevent potential complications associated with shipping. However, sediments may be encountered with characteristics that are outside of the tolerance range of one or more of the species. For example, grain size limitations for *A. abdita* and *R. abronius* are <10 % and >90 % fines, respectively. If these species are exposed to sediments that exhibit textural characteristics outside of these extremes, any mortality that is observed could be due to effects of grain size independent of contaminants associated with the sediment. Ambiguity in interpretation may be avoided by careful consideration of the test species given the sediment to be tested. Comparative information is available for the four species on sediment grain size sensitivity, salinity application ranges, and sensitivity to aqueous ammonia (section A2.4).

12.3 *Field Collection:*

12.3.1 Field collection is presently the most common method for obtaining estuarine or marine amphipods for sediment testing. All four species are commonly collected, shipped, and held in the laboratory; However, (USEPA-USACE 2001(2)) recommends establishing laboratory cultures of *L. plumulosus* (section 12.5). Commercial vendors are available for all four species. The availability of the appropriate size class for each species may vary seasonally. The collection site chosen should be one for which the presence of abundant organisms of the correct size and age has been demonstrated previously, and identification of the species has been confirmed taxonomically (for example, Bousfield, 1973 (168); Barnard and Barnard, 1982 (189)). Collection areas should be relatively free of contamination. All individuals in a test must be from the same source, because different populations may exhibit different sensitivities to contaminants. The four species are found in distinctly different habitats (Table 5).

12.3.2 *Species-specific Habitat Characteristics:*

12.3.2.1 *Ampelisca abdita* is found mainly in protected areas from the low intertidal zone to depths of 60 m. This species generally inhabits sediments from fine sand to mud and silt without shell fragments, although it can also be found in relatively coarser sediments with a sizeable fine component. This species is often abundant in sediments with a high organic content Aggregations of *A. abdita* are indicated by an abundance of tubes on the sediment surface, location of which can be facilitated by looking through a glass-bottom bucket. Although populations may be seasonally ephemeral, *A. abdita*

is routinely collected year-round for toxicity testing from subestuaries of Narragansett Bay, RI and from San Francisco Bay, CA.

12.3.2.2 *Eohaustorius estuarius* is found on protected and semi-protected beaches from mid-water level to shallow subtidal, within the upper 10 cm (Environment Canada, 1992 (**5**)). *Eohaustorius estuarius* can be found on open coasts in beds of freshwater streams flowing into the ocean, and in sand banks in estuaries, above the level of other regional eohaustorids (E. sawyeri and E. washingtonianus; Environment Canada, 1992 (**5**)). *Eohaustorius estuarius* inhabits clean, medium-fine sand with some organic content It is routinely collected for toxicity tests from Yaquina Bay OR and Beaver Creek near Newport, OR, and on the west coast of Vancouver Island, BC, Canada.

12.3.2.3 *Leptocheirus plumulosus* is found in subtidal portions of Atlantic Coast brackish estuaries. It is common in protected embayments, but has been collected in channels of estuarine rivers up to depths of 13 m. It is most often found in fine-grained sediment with a high proportion of particulate organic material, although it has been collected in fine silty sand with some organic content.

12.3.2.4 Primary habitats of *R. abronius* include nearshore subtidal zones on the Pacific Ocean coastline, and sub- and intertidal zones within polyhaline portions of estuaries in the Pacific Northwest. *Rhepoxynius abronius* naturally inhabits clean, fine sand. It has been collected for use in toxicity tests from Lower Yaquina Bay, OR (Swartz et al. 1985 (**10**)), and West Beach, Whidbey Island, WA (Ramsdell et al. 1989 (**194**); Word et al. 1989 (**17**)).

12.3.3 *Collection Methods:*

12.3.3.1 Subtidal amphipods can be collected with a small dredge or grab (for example, PONAR, Smith-McIntyre, or Van Veen). Intertidal populations can be collected using a shovel or by skimming the sediment surface with a long-handled, fine-mesh net. At least one-third more amphipods should be collected than are required for the test.

12.3.3.2 All apparatus used for collecting, sieving, and transporting amphipods and control-site sediment should be clean and made of non-toxic material. They should be marked "live only" and should never be used for working with formalin or any other toxic materials and should be stored separately from the aforementioned. The containers and other collection apparatus should be cleaned and rinsed with deionized water, dechlorinated laboratory water, reconstituted seawater, or natural seawater from the collection site or an uncontaminated source before use.

12.3.3.3 To minimize stress, amphipods should be handled carefully, gently, and quickly, and only when necessary. Amphipods can be isolated from collection-site sediment using gentle sieving. *Ampelisca abdita* is exceptional in requiring vigorous sieving to induce the animals to leave their tubes. Once sieved, attempts should be made to keep amphipods submersed in collection site sea water at the ambient collection temperature at all times. Amphipods that are dropped, or injured should be discarded. Once separated from the sediment, amphipods should not be exposed to direct sunlight.

12.3.3.4 The mesh size of the sieve will depend on the species collected. Sieves with 0.5-mm mesh should be used for sediment containing *A. abdita* and *L. plumulosus*. Larger *A. abdita*, which should not be used in the test, should be excluded by sieving first with a 1.0-mm screen. When sieving *A. abdita*, only about half of the amphipods will be extracted from their tubes. The tube mat should be placed undisturbed for 20 to 30 min to coax the remaining animals out. Sieves with 1.0-mm mesh should be used for *E. estuarius* and *R. abronius*.

12.3.3.5 Collection-site water should be used to sieve sediment in the field. A 2-cm thick layer of sieved collection site sediment should be placed in transport containers, and this sediment covered with collection-site water. Detritus and predators recovered by sieving should be removed, and the collected amphipods should be gently washed into the transport containers with collection site water.

12.3.3.6 The salinity and temperature of surface and bottom sea water at the collection site should be measured and recorded. An adequate portion of collection site sediment should be returned with the amphipods to serve as both laboratory holding sediment or for use as control sediment in the toxicity test.

12.3.3.7 During transport to the laboratory, amphipods should be kept in sieved collection-site sediment at or below the collection site temperature. Containers of amphipods and sediment should be transported to the laboratory in coolers with icepacks, and the water in the containers of amphipods should be aerated if transport time exceeds 1 h.

12.3.3.8 An alternate collection method for *A. abdita* involves transporting intact field-collected tubes to the laboratory for isolation of amphipods. This method is advantageous because separation of *A. abdita* from its tubes may be time-consuming when attempted in the field, a practice which may be impractical in cold winter months. Amphipod tubes are collected and placed on a 0.5-mm sieve. The sieve should be shaken vigorously to remove most of the sediment, leaving the intact tubes. The tubes should be placed into a covered bucket that contains a sufficient quantity of collection site water to cover the collected material, and transported to the laboratory. In the laboratory, the tubes should be removed from the collection buckets and placed on a sieve series consisting of a 2-mm mesh sieve over a 0.5-mm mesh sieve. Amphipods should be forced from their tubes by spraying sea water on the material present on the 2-mm sieve. When all the tube material has been sprayed, the 0.5-mm sieve should be shaken vigorously to separate amphipods from any material that is present. The 0.5-mm sieve should then be completely submersed, at which point the amphipods will float on the water surface. The amphipods should then be skimmed from the surface with a small aquarium net and transported to a container with sea water at the appropriate temperature. The shaking process should be continued until only a few amphipods remain in the sieve.

12.3.3.9 *Leptocheirus plumulosus*—Although established cultures of *L. plumulosus* are the recommended source of organisms for new cultures, it is recognized that field collection of amphipods might be necessary to enhance genetic diversity of existing cultures or to establish new cultures at a laboratory

(USEPA-USACE 2001 (2)). The taxonomy of the organisms should be confirmed before they are introduced into existing laboratory populations. New organisms should be carefully inspected, and all other species of amphipods should be removed. The ability of a wild population of sexually reproducing organisms to crossbreed with existing laboratory populations of *L. plumulosus* should be confirmed through long-term culture maintenance (Duan et al., 1997 (170)). Collection areas should be relatively free of contamination. Field collection of *L. plumulosus* neonates for immediate use in a chronic toxicity test is not recommended.

12.3.3.10 *L. plumulosus* is subtidal and can be collected with a small dredge or grab (for example, Ponar, Smith-McIntyre, or Van Veen). In very shallow water, sediment containing *L. plumulosus* can be collected with a shovel or scoop, or using a suction dredge (DeWitt et al., 1992a (6)). *L. plumulosus* can be isolated easily from collection-site sediment by gentle sieving. Ideally, amphipods will be separated into adults, subadult, and neonates. To reduce field processing time, 1.0-mm and 0.6-mm mesh sieves can be used to isolate adults and subadults with which to start a culture. Sediment passing through the 0.6-mm sieve could be temporarily used for holding until further processing of the sediment is practical. The final sieving of collection-site sediment through 0.25-mm mesh can be deferred until materials are returned to the laboratory.

12.3.4 *Life Stage and Size:*

12.3.4.1 The life stage for amphipods used in sediment toxicity tests will depend on the species tested. For *A. abdita* and *L. plumulosus*, sub-adult (immature) individuals should always be selected for testing. The life cycle of these species is relatively short, so the likelihood of senescence and any effects that could be associated with reproductive development or maturation are minimized if young individuals are selected. *Eohaustorius estuarius* and *R. abronius* are annual species with longer life spans than *A. abdita* and *L. plumulosus*. Mature individuals can be used providing they are within the recommended size range.

12.3.4.2 The size range of test animals should be kept to a minimum regardless of the chosen species. For all species, mature female amphipods, which are distinguishable by the presence of embryos in the brood pouch or oviduct, should not be selected for testing. Additionally, mature male *A. abdita* and *L. plumulosus* should not be used. Recommended size ranges for the four species are as follows:

(1) Ampelisca abdita—3 to 5 mm; or those amphipods retained on a 0.71-mm sieve after passing through a 1.0-mm sieve. Adult male animals should not be tested; they are active swimmers and die shortly after mating.

(2) Eohaustorius estuarius—3 to 5 mm; or those amphipods retained on a 1.0-mm sieve. Large individuals (that is, >5 mm) should not be tested because they might be senescent.

(3) Leptocheirus plumulosus—For 10-d toxicity testing: 2 to 4 mm; or those amphipods retained on a 0.5-mm sieve after passing through a 0.71-mm sieve. See section 12.5 for methods to obtain *L. plumulosus* from cultures to start a 28-d sediment exposure.

(4) Rhepoxynius abronius—3 to 5 mm; or those amphipods retained on a 1.0-mm sieve. Large individuals (that is, >5 mm) should not be tested because they might be senescent.

12.3.5 *Shipping Methods:*

12.3.5.1 All four species have been routinely shipped from the collection site to the laboratory for sediment toxicity testing. Currently, shipping from the collection site is necessary for many testing laboratories because culture methods are not available for all four species. It is important that shipping methods ensure that consistently healthy animals are used in successive toxicity tests. Additionally, the amphipods that are received by a laboratory should meet the shipping acceptance criteria recommended for each species. Shipping methods and acceptance criteria will vary depending on the species used.

12.3.5.2 *Ampelisca abdita*—Collected amphipods should be shipped within 24 h of collection. Acceptable methods are available for shipping *A. abdita* in sediment and in water. For shipping in sediment, small plastic "sandwich" containers (about 500 mL) with sealable lids should be used. The containers are filled three-quarters full with a minimum depth of 2 cm of sieved fine-grain collection-site sediment and then to the top with well-aerated seawater. No more than 200 amphipods should be added to each container. Amphipods should be allowed to burrow into the sediment and build tubes before the containers are sealed. Containers should be sealed with lids under water to eliminate any air pockets. For shipping in water-only, scalable plastic bags 60 (about 1 L) should be used. Amphipods in their tubes should be placed in bags and a sufficient amount of collection site water should be added to keep the tubes moist. The air in the bag should be replaced with pure oxygen before sealing, and then placed into a second bag. Bags should be placed in a container that has a layer of material (that is, styrofoam or newspaper) sufficiently thick to prevent excessive movement over a layer of ice-packs. The shipping container should be marked to prevent it from being inverted.

12.3.5.3 *Eohaustorius estuarius and Rhepoxynius abronius*—Shipping methods for these organisms are essentially the same. Small plastic "sandwich" containers (about 500 mL) with scalable lids should be used. The containers are filled three-quarters full with sieved collection site sediment (fine sand) and then with a 1-cm layer of collection site sea water. Not more than 100 amphipods should then be added and allowed to burrow. After the animals have burrowed, the overlying water should be poured off, but the sediment should be moist. The containers are then sealed and ready for shipment.

12.3.5.4 *Leptocheirus plumulosus— L. plumulosus* should be shipped in water only (USEPA-USACE 2001 (2)). Care should be taken to select containers with a firm seal that is not easily broken in shipment. The containers are filled to the top with well-aerated water. No more than 100 amphipods/L should be added to each container. For shipping, sealable plastic bags, cubitainers, and other sealable plastic containers can be used. The containers should be filled with well-aerated collection-site water or culture water before they are sealed. The double packing bags should be placed in a container that has a protective layer of material (that is, Styrofoam or newspaper) sufficiently thick to prevent excessive movement

with an under layer of ice packs. The shipping container should be marked to prevent it from being inverted.

12.3.6 *Performance Criteria for Shipped Amphipods:*

12.3.6.1 The process of ensuring the availability of healthy amphipods on the day that the test is set up begins when the animals arrive in the laboratory from the supplier. Although the ultimate performance criterion for amphipods utilized in sediment toxicity tests is achievement of >90 % survival in control sediment (Table A1.3 in Annex A1), it would be desirable to assess the quality and acceptability each batch of shipped amphipods using the criteria that follow. For all four species, biological criteria should include an exhibition of active swimming behavior upon placement in water, full digestive tracts, and an acceptable color. *Ampelisca abdita* should be opalescent pink, *E. estuarius* should be grayish- or yellowish white, *L. plumulosus* should be brown or orange-gray, and *R. abronius* should be salmon pink, grayish- or yellowish-brown, or white with a pinkish-brown hue. Mortality among the shipped animals should not exceed 5 %. No sexually mature animals should be included in shipments of *A. abdita* or *L. plumulosus*. The shipping containers should arrive intact, and the temperature of water or sediment in shipping containers should be between 4 and 10°C. Information on physical parameters of the collection site, including at least temperature and salinity, should be provided by the supplier. Finally, a quantity of collection site sediment should be included as substratum for amphipods during the acclimation period or for use as control sediment in the test. It may be desirable for the testing facility to stipulate these criteria to the supplier when the animals are ordered. If these criteria are not met, the animals may have experienced stress during shipment, and >90 % survival in control sediment may not be achieved.

12.4 *Holding and Acclimation:*

12.4.1 *Density*—Amphipods should be held and acclimated (if necessary) in containers that contain a 2- to 4-cm layer of collection site sediment that has been sieved through a 0.5-mm mesh screen. Amphipod density should not exceed 1 amphipod/cm^2. *Ampelisca abdita* that have been shipped in their tube material in bags can be held under those conditions as long as aeration and food are supplied and the temperature and salinity holding and acclimation procedures are followed.

12.4.2 *Duration*—Depending on temperature and salinity at the collection site, amphipods may have to be acclimated to test conditions. If necessary, changes in temperature or salinity to bring amphipods from the collection site conditions to the test conditions should be made gradually (for example, should not exceed 3°C and 5 ‰ per 24 h). Once test conditions are achieved, amphipods should be maintained at these conditions for at least two days before testing to allow for acclimation. Amphipods held for more than ten days should not be used for testing because they may not satisfy performance control criteria. Temperature and salinity should be measured at least daily during the period when amphipods are being adjusted to the conditions of the test water. Thereafter, temperature, salinity, pH, and DO should be measured in the holding containers at least at the start and end of the acclimation period, and preferably daily.

12.4.3 *Temperature*—Overlying water temperature should not be changed by more than 3°C per day during acclimation to the test temperature. Once the test temperature is reached, amphipods should be maintained at that temperature for a minimum of 2 d. A water bath, an incubator, or temperature-regulated room can be used for temperature acclimation.

12.4.4 *Salinity*—It is unlikely that either *A. abdita* or *R. abronius* will require salinity acclimation because the collection site salinity for these two species will likely be within 3 ‰ of the test salinity of 28 ‰ . Salinity of water used for temperature acclimation for these species, if necessary, should be the test salinity, or 28 ‰ . The target test salinity for *E. estuarius* and *L. plumulosus* is 20 ‰, and it is likely that the collection site salinity will be considerably lower than this for both species. Upon arrival in the laboratory, the water used to hold *E. estuarius* and *L. plumulosus* should be adjusted to 20 ‰ by adjusting the salinity in the holding container at a rate that should not exceed 5 ‰ per 24 h. The amphipods should be maintained at 20 ‰ for 2 d before testing. A salinity of 5 or 20 ‰ is recommended for routine application of 28-d test with *L. plumulosus* (Annex A2; USEPA-USACE 2001 (**2**)) and a salinity of 20 ‰ is recommended for routine application of the 10-d test with *E. estuarius* or *L. plumulosus* (Annex A1). However, the salinity of the overlying water for tests with these two species can be adjusted to a specific salinity of interest (for example, salinity representative of site of interest or the objective of the study may be to evaluate the influence of salinity on the bioavailability of chemicals sediment). Importantly, the salinity tested must be within the tolerance range of the test organisms (as outlined in Annex A1 and Annex A2). If tests are conducted with procedures different from those described section 1.3 or in Table A1.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (section 1.10).

12.4.5 *Lighting*—Lighting should be constant and continuous throughout the holding and acclimation period for 10-d tests, all species except *A. abdita* require a 16:8 L:D photoperiod to promote feeding. Fluorescent lights should be used, and they should provide from 500 to 1000 lux at the surface of the sediment in holding containers.

12.4.6 *Water:*

12.4.6.1 Provided that it is acceptable to the test organisms, either an uncontaminated supply of natural sea water or reconstituted sea water can be used for holding and acclimation (section 7.1). At a minimum, healthy amphipods should exhibit acceptable survival in holding water, and should not exhibit signs of stress, such as unusual behavior or changes in appearance.

12.4.6.2 If natural sea water is used, it should be obtained from an uncontaminated area known to support a healthy, reproducing population of the test species or comparatively sensitive species. Reconstituted sea water is prepared by adding commercially available sea salts to water from a suitable source, in quantities sufficient to provide the desired salinity. Pre-formulated brine (for example, 60 to 90 %) prepared with dry ocean salts or heat- concentrated natural sea water can also be used. To obtain the desired holding or

acclimation salinity, sea salts or brine can be added to a suitable fresh water, natural estuarine water, or the laboratory's sea water supply. The suitability and consistency of a particular salt formulation for use in holding and acclimation should be verified by laboratory tests because some formulations can produce unwanted toxic effects or sequester contaminants (Environment Canada, 1992 (**5**)). Reconstituted water should be intensively aerated for two weeks before use (Environment Canada, 1992 (**5**) ; section 7.1). Suitable sources of water used for preparing reconstituted sea water include deionized water or an uncontaminated natural surface water or ground water. Chlorinated water must never be used because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals. Dechlorinated municipal drinking water should be used only as a last resort because dechlorination is often incomplete.

12.4.6.3 Assessments of the quality of the water used for holding and acclimation and for preparing reconstituted sea water should be performed as frequently as required to document acceptability (section 7.1). Analyses of variables including salinity, temperature, suspended solids, pH, DO, total dissolved gasses, ammonia, nitrite, pesticides, and metals are recommended. Sea water used for holding and acclimating amphipods should be filtered (<5 μm) shortly before use to remove suspended particles and organisms and should be aerated for a two weeks before use (section 7.1).

12.4.6.4 *Feeding—Ampelisca abdita* and *L. plumulosus* require supplemental feeding during holding or acclimation for 10-d toxicity tests conducted with field-collected organisms. *Ampelisca abdita* should have food available daily, whereas *L. plumulosus* should be fed every other day. *Ampelisca abdita* can be supplied with an algal ration consisting of *Pseudoisochrysis paradoxa* or *Phaeodactylum tricomutum* that is provided in conjunction with sea water renewal. Some laboratories have reported success in providing *A. abdita* enriched dried algal material (Docosa Gold and Golden Shell algal-based natural feed supplements (Sanders Brine Shrimp Company, Ogden, Utah)) slurried in seawater (Michele Redmond, Northwestern Aquatic Sciences, Newport, OR; personal communication). Other diets can be used provided the diet has been demonstrated to support acceptable organisms for testing (Table A2.3 in Annex A2). See Stein (1973 (**195**)) for procedures to culture algae. After 75 % of the overlying water has been removed, each holding container should be renewed with sea water at the appropriate salinity that contains algae at a concentration of at least 1×10^6 cells/mL. *Leptocheirus plumulosus* should also be provided with dry food ration, consisting at a minimum of finely powdered Tetramarine[4] (formerly called TetraMin[4]). It may be desirable to grind the dry food in a blender. Each container should receive about 0.4 g dry food/350 amphipods. *Eohaustorius estuarius* and *R. abronius* will utilize organic material in the holding sediment as food and do not require supplemental feeding.

12.4.6.5 *Acceptability of Animals*—Amphipods counted into the holding or acclimation chambers should be active and appear healthy. Any individuals that fail to burrow or fail to make tubes (that is, *A. abdita*) in holding sediment or that appear unhealthy during the holding or acclimation period should be discarded. Apparently dead individuals should also be discarded. If greater than 10 % of the amphipods emerge or appear unhealthy during the 48 h preceding the test, the entire group should be discarded and not used in the test. Additionally, the group should be discarded if more than 10 % of the amphipods die or become inactive during the holding period before testing.

12.5 *Culture Procedure for Leptocheirus plumulosus:*

12.5.1 *General Culturing Procedures:*

12.5.1.1 Acceptability of a culturing procedure is based in part on performance of organisms in culture and in the sediment test (Table A2.3 in Annex A2). No single technique for culturing test organisms is required. What may work well for one laboratory may not work as well for another laboratory. Although a variety of culturing procedures are outlined below for *L. plumulosus*, organisms must meet the test acceptability requirements listed in Table A2.3 of Annex A2.

12.5.1.2 All organisms in a test must be from the same source. 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.

12.5.1.3 Obtaining organisms from wild populations should be avoided unless organisms are cultured through several generations in the laboratory before use in testing. In addition, the ability of the wild population of sexually reproducing organisms to crossbreed with the existing laboratory population should be determined (Duan et al., 1997 (**170**)).

12.5.1.4 Test organisms obtained from commercial sources should be shipped in well-oxygenated water without sediment in insulated containers to maintain temperature during shipment. Temperature, salinity and DO of the water in the shipping containers should be measured at the time of shipment and on arrival to determine if the organisms might have been subjected to low DO, salinity change, or temperature and salinity fluctuations. The temperature and salinity of the shipped water should be gradually adjusted to the desired culture temperature and salinity at rates not exceeding 3°C or 3 ‰ per 24 h.

12.5.2 The culturing method below is based on procedures described in DeWitt et al. (1997a (**7**)). A periodic-renewal culture system is used. It consists of culture bins that contain aerated water over a thin (about 1 cm) layer of clean, fine-grained sediment in which the amphipods burrow. Culturing areas must be separate from testing areas to avoid exposing the cultures to contaminants. Before *L. plumulosus* are received at a testing facility, appropriate permits or approvals for import of live organisms should be obtained, if necessary. If culturing is to occur in an area where *L. plumulosus* are not indigenous to local waters, precautions should be taken to prevent release of living organisms to the outside environment (section 10.5.15). Test animals should be destroyed at the end of toxicity test.

12.5.3 *Starting a Culture*—Amphipods for starting a laboratory culture of *L. plumulosus* should be obtained from a source with an established culture in which the species has

[4] Tetramarine is a trademark of Tetra Werke, Melle, Germany.

been verified for commercial sources of *L. plumulosus*). Alternatively, *L. plumulosus* can be obtained from field populations (section 12.3). Upon receipt of amphipods, the temperature and salinity of the water in shipping container(s) should be gradually adjusted to 20°C and desired culture salinity, at rates not exceeding 3°C or 3 ‰ per 24-h period. Feeding and regular maintenance should begin once the acclimation period is complete. Separate organisms into three size classes by gentle sieving: adults (retained on 1.0-mm mesh), subadults (pass through 1.0-mm mesh and retained on 0.6-mm mesh), and neonates (pass through 0.6-mm mesh and retained on 0.25-mm mesh). Seed each culture bin with about equal numbers of adults, subadults, and neonates to achieve a population density between 0.25/cm^2 to 0.35/cm^2 (2500 to 3500/m^2). Select only actively moving, healthy-looking organisms. Cultures should not be stocked at densities greater than 0.5/cm^2 (5000/m^2). See section 12.5.13.3 for guidance on maintaining culture densities. Field-collected organisms should be added periodically to the culture population to maintain genetic diversity of the cultured amphipods.

12.5.4 *Culture Bins*—Culture bins should be easy to maintain. Plastic wash tubs (about 35 by 30 by 15 cm) have been used successfully by several laboratories (DeWitt et al., 1992a (**6**)). They are relatively light when filled with water and sediment, broad enough to allow for easy viewing of amphipod burrows, easily cleaned, inexpensive, and readily available. A wide variety of containers and materials may work just as well for culturing this species. New plasticware should be soaked in running water for several days before use in the cultures to leach out potentially toxic compounds. Previously used culture bins usually can be satisfactorily cleaned using hot water and a scrub brush or pad, without the use of a chemical cleanser. Culture bins should not be washed with soap or detergent except in extreme conditions. If such a cleaning is deemed necessary, culture bins should be rinsed and soaked thoroughly after cleaning to remove residual cleanser.

12.5.5 *Culture Sediment*—Cultures should be established with a thin layer (1 to 1.5 cm) of sediment spread on the bottom of a culture bin. Sediment used for culture purposes can be the same as the control sediment used in sediment toxicity tests. Suitable sources for culture sediment include the amphipod collection site or an area adjacent to salt marsh vegetation. Culture sediment should be uncontaminated, organic-rich, fine-grained marine or estuarine sediment that is not anoxic. The organic carbon content (% TOC) should range between 1.5 % and 4 %. The sediment should be press-sieved through a 0.25-mm screen before use to facilitate the harvesting of neonates and removal of indigenous macroinvertebrates. Culture sediment can also be wet sieved. Wet-sieving involves agitating or swirling the sieve containing sediment in water so that particles smaller than the selected mesh size are washed through the sieve into a container. The sieve may be placed on a mechanical shaker, or the sediments on the screen can be stirred with a nylon brush to facilitate the process. Alternatively, the particles may be washed through the sieve with a small volume of running water. Culture sediment can also be frozen (>48 h) to provide additional assurance that viable macroinvertebrates are not present. Frozen sediment should be homogenized after thawing and before use. Culture sediment can be stored frozen for about 1 year.

12.5.6 *Culture Water*—Culture water used for holding and acclimating test organisms and for conducting toxicity tests should be of uniform quality and from the same source. See section 7.1 for acceptable sources of water. Cultures of *L. plumulosus* are usually maintained at a salinity of either 5 ‰ or 20 ‰ . Culture salinity will depend on the anticipated pore-water salinity of test sediment and desired overlying water salinity to be used in the test (Table A2.1 in Annex A2). Alternatively, the salinity of the overlying water can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest; section 1.4). To obtain these salinity values, natural or reconstituted seawater should be diluted with nonchlorinated well water, deionized water, or reverse-osmosis water. Seawater and dilution water should be filtered (<5 µm). Water that might be contaminated with pathogens should be treated shortly before use by filtration (<0.45 µm), either alone or in combination with ultraviolet sterilization. DO, salinity, and pH should be checked before the water is used in cultures. Batches of salinity-adjusted culture water can be held for about 1 week; a lower holding temperature (<20°C) helps maintain acceptable water quality. Water depth in culture bins should be at least 10 cm. Aeration, provided through an air stone or pipette, should be moderate and constant, but not so vigorous as to resuspend sediment. Overlying water should be replaced the day after a new culture is established; thereafter, it should be renewed two or three times per week.

12.5.7 *Temperature and Photoperiod*—Cultures should be maintained at 20 to 25°C. The reproductive rate of *L. plumulosus* increases at temperatures greater than 20°C, necessitating more frequent culture thinning. Higher temperatures also can promote unwanted growth of nuisance organisms (such as nematodes, small worms, copepods). Temperatures below 20°C may not foster sufficiently prolific reproductive rates. Fluorescent lights should be on a 16 h light : 8 h dark photoperiod at a light intensity of 500 to 1000 lux. An efficient procedure is to maintain long-term cultures at 20°C, and increase culture temperature to about 25°C a few weeks in advance of testing.

12.5.8 *Food and Feeding*—This method recommends the simplest effective diet for routine use for *L. plumulosus* culture by providing finely milled Tetramarine[4] two or three times per week. Tetramarine[4] is a dry fish food (flake or powder) widely available in retail pet stores. The food is prepared by milling, grinding, or chopping the flakes to a fine powder. A small flour mill, blender, or coffee grinder is useful for this. Ground powder is then sifted through a 0.25-mm mesh screen, retaining and using only the material that passes through the sieve. Use of a respirator or fume hood will minimize aspiration of dust. When establishing a new culture bin, do not add food for 3 to 4 days after amphipods are placed in new sediment. This will encourage the organisms to consume labile organic matter in sediment and to turn over the sediment by burrowing and feeding.

12.5.8.1 Culture bins should be provided with food in conjunction with water renewal. Two or three times a week, about 60 % of culture water should be removed from each culture bin (by decanting, siphoning, or pumping) and replaced with the same volume of renewal water. Each culture bin is provided with about 0.4 g of dry food sprinkled evenly over the water surface, or as a slurry in culture water two or three times per week (for example, Monday-Wednesday-Friday or Monday-Thursday). The amount of dry food added will depend on the density of each culture bin. Newly started culture bins should receive slightly less food (for example, 0.3 g) than bins containing mature cultures. Excess food can decompose encouraging microbial and fungal growth on the sediment surface deteriorating water quality.

12.5.8.2 Some laboratories have experienced success in culturing *L. plumulosus* when other food is provided (that is, live microalgae or a mixed dried food; DeWitt et al., 1992a (**6**)). Modifications to the diet can be used by laboratories in order to optimize culture practices as long as performance criteria are satisfied (Table A2.3 in Annex A2).

12.5.8.3 One feeding alternative is to supply renewal water consisting of seawater, cultured phytoplankton, and deionized water combined to the proper salinity and adjusted to an algal density of about 10^6 cells/mL (DeWitt et al. 1992a (**6**)). Proportions will vary depending upon the salinity of the seawater and the density of the cultured phytoplankton. Live algae also can be used periodically to supplement a routine supply of dry food. The algae used can include a single or multiple species (for example, *Pseudoisochrysis paradoxa*, *Phaeodactylum tricornutum*, *Isochrysis galbana*, *Chaetoceros calcitrans*, *Skeletonema sp.*, *Dunalicella tertiolecta*, and/or *Thallasiosirus spp.*). Other algal species might be used if it can be demonstrated that they foster amphipod growth and reproductive rates equal to those of the aforementioned food alternatives. A mixture of algal species is recommended, if live algae is included in the diet.

12.5.9 *Culture Maintenance*—Cultures should be observed daily to ensure that temperature is acceptable and aeration is adequate in all culture bins. Inspection for the presence of oligochaetes, polychaetes, copepods, infaunal sea anemones, or chironomids should be conducted weekly. The presence of excessive densities of these or other competing or predatory organisms should prompt renewal of culture sediment after separating *L. plumulosus* from the invasive organisms. During routine maintenance, cultures should be inspected for the presence of microbial and fungal build-up on the sediment surface. This build-up appears as a white or gray growth that may originate near uneaten food. Presence of microbial build-up may indicate that more food is being provided than is required by the amphipods. No additional food should be provided to culture bins with surficial microbial build-up until the build-up is no longer present. Sieving of sediment and renewal of culture bins can expedite removal of decaying material.

12.5.9.1 Healthy cultures are characterized by an abundance of burrow-openings on the sediment surface and turbid water from amphipod activity. Although amphipods may leave their burrows to search for food or mates, they will ordinarily remain in their burrows during the illuminated portion of the photoperiod. Amphipod density should therefore only be estimated by examining the number of burrow openings. An abundance of organisms on the sediment surface (for example, >15 per culture bin) could indicate inadequate sediment quality, low DO concentrations, or overcrowding. A culture bin with an abundance of amphipods or unhealthy individuals on the sediment surface should be examined closely, and the DO concentration should be measured in the overlying water. If the DO concentration is below 60 % saturation (<4.4 mg/L), the culture bin should be sieved, and the population and culture sediment examined. If the population is too dense (that is, >1.5/cm^2), the culture should be thinned. If the sediment becomes an unacceptable habitat because it is anaerobic or black and sulphidic below the sediment surface, or contains an excess of competitive or predatory organisms, the healthy surviving amphipods should be placed in a new culture bin with newly prepared culture sediment.

12.5.9.2 Water temperature and DO should be measured in culture bins on a regular basis, about every week. Cultures should be continuously aerated. Salinity should be measured after water renewal. Ammonia and pH in overlying water should be measured with each new batch of sediment before organisms are added.

12.5.9.3 *Renewal of Cultures— L. plumulosus* can be prolific, and care should be taken to ensure that culture bins do not get overcrowded. Amphipods in overcrowded culture bins can be stressed because of food and space limitations, causing the fecundity of females to drop below five eggs/brood/female and the abundance of neonates and subadults to decline dramatically. Culture density should not exceed 1.5 amphipods/cm^2 and should ideally be maintained at about 0.5 amphipods/cm^2. A typical indication of overcrowding is a fairly uniform size distribution of amphipods (mostly small adults) and the presence of only two to four eggs in the brood pouches of gravid females. If sediment is not replaced occasionally, the cultures may become infested with undesirable species, such as worms or copepods. These “pests” may compete for food, bind sediment as fecal pellets, or produce mucus, thereby reducing culture productivity or increasing the effort required to harvest amphipods. Field-collected organisms should be added to the culture population periodically (about annually) in order to maintain genetic diversity of the culture organisms.

12.5.9.4 To avoid overcrowding, cultures should be thinned every 6 to 8 weeks by sieving through a 0.25-mm mesh screen to remove sediment. Sediment can be used for a total of 2 to 4 months before it should be replaced. Discard old sediment, prepare new culture bins and sediment, and restock each bin.

12.5.10 *Obtaining Leptocheirus plumulosus for a 10- and 28-d Test:*

12.5.10.1 The cultures usually can be harvested about 4 to 5 weeks after initiation or up until the cultures are thinned and renewed (6 to 8 weeks after initiation). Neonates used for testing may be selected on the basis of size or age. For size-selected neonates, the contents of culture bins are gently sieved through 0.60-mm and 0.25-mm screens. Neonates used for testing in 28-d tests may be selected on the basis of size or

age. For size selected organisms, animals passing through a 0.6 or 0.5 mm sieve onto a 0.25 mm mesh are used for testing and individual neonates typically have a dry weight of about 0.03 mg to 0.06 mg and body length of about 1.3 mm to 1.7 mm. In contrast, *L. plumulosus* used in the 10-d test are those that pass through a 0.71 mm sieve on to a 0.5 mm sieve. Culture bins of about 35 by 30 cm typically produce at least 300 to 400 neonates with a healthy culture. Selecting neonates for testing based on size is the preferred option for method comparability. For age-selected neonates, gravid females are isolated from cultures 5 d before test initiation. Gravid females are placed in separate culture bins with sediment and are fed. Two days before test initiation, these females are then transferred to bins containing only water (for example, at 25°C and 5 ‰ or 20 ‰ %). On the day of test initiation, the contents of these bins are gently passed through a 1-mm screen on which adults are retained. Neonates that pass through this screen are transferred to a shallow glass container for sorting. Special care should be taken to ensure that the neonates are handled gently, selecting and transferring them with wide-bore pipits only, and maintaining the water temperature and salinity within recommended test conditions.

12.5.10.2 About one-third more amphipods than are needed for the test should be sieved from the sediment and transferred to a sorting tray. The additional organisms allow for the selection of healthy, active individuals. Organisms not used in toxicity tests can be used to establish new cultures.

12.5.10.3 Amphipods placed in the holding bins should be active and healthy. Sluggish or apparently dead individuals should be discarded. If greater than 10 % of the amphipods in the holding bins appear unhealthy or are dead during 48 h preceding the test, the entire group should be discarded and not used in tests.

12.5.11 *Minimization of Risk of Release of Nonindigenous Organisms:*

12.5.11.1 If test amphipods are not endemic to the local estuarine environment, containment and water treatment procedures should be implemented to minimize the chance of accidental release of organisms or pathogens to local waters. The same precautions might also be required if the culture population of *L. plumulosus* is not derived from local sources. Some local or state authorities might require special permits and procedures to allow receipt and culturing of nonindigenous species. All test animals should be destroyed at the end of toxicity tests. Culturing and holding of the amphipods should only occur in specially designated laboratory areas, separate from those used to hold, culture, or experiment with native species. These areas should have no access to drains leading directly to local surface waters. Handling of nonindigenous species should be limited to trained and authorized personnel. The amphipods should be cultured in a static-renewal manner to minimize the amount of water that needs to be treated. Any seawater removed from culture bins should be treated with chlorine bleach or ozonation to kill any escaping organisms and pathogens. All equipment used to culture or handle the amphipods should be cleaned thoroughly. Any excess or dead amphipods should be placed in bleach or treated by ozonation or heat killed (boiling water) to ensure they are killed before disposal as sanitary waste.

13. Calculation

13.1 *Data Recording:*

13.1.1 Quality assurance project plans with data quality objectives and standard operating procedures should be developed before starting a test. Procedures should be developed by each laboratory to verify and archive data (USEPA 1994e, **(196)**).

13.1.2 A file should be maintained for each sediment test or group of tests on closely related samples (Section 11). This file should contain a record of the sample chain-of-custody; a copy of the sample log sheet; the original bench sheets for the test organism responses during the sediment test(s); chemical analysis data on the sample(s); control data sheets for reference toxicants; detailed records of the test organisms used in the test(s), such as species, source, age, date of receipt, and other pertinent information relating to their history and health; information on the calibration of equipment and instruments; test conditions used; and results of reference-toxicant tests. Original data sheets should be signed and dated by the laboratory personnel performing the tests. A record of the electronic files of data should also be included in the file.

13.2 *Data Analysis:*

13.2.1 Statistical methods are used to make inferences about populations, based on samples from those populations. In most sediment tests, test organisms are exposed to chemicals in sediment to estimate the response of the population of laboratory organisms. The organism response to these sediments is usually compared with the response to a control or reference sediment. In any sediment test, summary statistics such as means and standard errors for response variables (for example, survival, chemical concentrations in tissue) should be provided for each treatment (for example, pore-water concentration, sediment concentration). See Section 14 of Test Method E1706 and Guide E1847 provide specific guidance on statistical analyses of data from sediment tests. Specifically, Test Method E1706 provides guidance on the following: *(1)* experimental design (including replication, minimum detectable differences, randomization, pseudoreplication, compositing of samples) and *(2)* Statistical analysis of data (including hypothesis testing (for example, Analysis of variance) and regression analysis (for example, Effect concentrations (ECx) and Inhibition concentrations (ICx)).

13.2.2 *Types of Data*—Two types of data can be obtained from sediment tests. The most common endpoint in toxicity testing is mortality, which is a dichotomous or categorical type of data. Other endpoints might include growth and reproduction. These types of endpoints are representative of continuous data.

13.2.3 *Sediment Testing Scenarios*—Sediment tests are conducted to determine whether contaminants in sediment are harmful to benthic organisms. Sediment tests are commonly used in studies designed to: *(1)* evaluate hazards of dredged material, *(2)* assess site contamination in the environment (for example, to rank areas for cleanup), and *(3)* determine effects of specific contaminants, or combinations of contaminants,

through the use of sediment spiking techniques. Each of these broad study designs has specific statistical design and analytical considerations, which are described as follows.

13.2.3.1 *Dredged Material Hazard Evaluation*—In these studies, n (number) sites are compared individually to a reference sediment. The statistical procedures appropriate for these studies are generally pairwise comparisons. Additional information on toxicity testing of dredged material and analysis of data from dredged material hazard evaluations is available in (**114**, **115**, **116**).

13.2.3.2 *Site Assessment of Field Contamination*—Surveys of sediment toxicity are often included in more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlation can be improved and costs may be reduced if subsamples are taken simultaneously for sediment tests, chemical analyses, and benthic community structure determinations. There are several statistical approaches to field assessments, each with a specific purpose. If the objective is to compare the response or residue level at all sites individually to a control sediment, then the pairwise comparison approach described as follows is appropriate. If the objective is to compare among all sites in the study area, then a multiple comparison procedure that employs an experiment-wise error rate is appropriate. If the objective is to compare among groups of sites, then orthogonal contrasts are a useful data analysis technique.

13.2.3.3 *Sediment Spiking Experiments*—Sediments spiked with known 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, EC50, IC50, NOEC, or LOEC. The statistical approach for spiked sediment toxicity tests also applies to the analysis of data from water-only reference-toxicity tests (Test Method E1706).

13.2.4 *Experimental Design*—The guidance outlined below on the analysis of data is adapted from a variety of sources including Test Method E1706, Guide E1688, Guide E1847, USEPA 1979b, 1993c, 1993b, (**131**, **159**, **165**), USEPA-USACE 1977, 1991, 1998 (**114**, **115**, **116**), Practices E29, E105, E122, E178, E141, and Terminologies E456, E1325, and E1402. The objectives of a sediment test are to quantify contaminant effects on or accumulation in test organisms exposed to natural or spiked sediments or dredged materials and to determine whether these effects are statistically different from those occurring in a control or reference sediment. Each experiment consists of at least two treatments: the control and one or more test treatment(s). The test treatment(s) consist(s) of the contaminated or potentially contaminated sediment(s). A control sediment is always required to ensure that no contamination is introduced during the experimental setup and that test organisms are healthy. A control sediment is used to judge the acceptability of the test. Some designs will also require a reference sediment that represents an environmental condition or potential treatment effect of interest. Controls are used to evaluate the acceptability of the test (Annex A1 and Annex A2) and might include a control sediment or a formulated sediment (section 7.2). 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 (that is, grain size, organic carbon) may be useful in these evaluations.

13.3 *Data Calculations:*

13.3.1 Sediments spiked with known 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 an NOEC (no observed effect concentration) or LOEC (lowest observed effect concentration). Most studies with spiked sediment are often started only a few days after the chemical has been added to the sediment. Consistent spiking procedures should be followed in order to make interlaboratory comparisons (section 10.3).

13.3.2 Evaluating effect concentrations for chemicals in sediment requires knowledge of factors controlling the 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 (DiToro et al. 1991, USEPA 1992c(**69**, **94**)). 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 are often inversely correlated with the organic carbon concentration of the sediment. Whatever the route of exposure, the correlations of effect concentrations to interstitial water concentrations indicate predicted or measured concentrations in interstitial water can be useful for quantifying the exposure concentration to an organism. Therefore, information on partitioning of chemicals between solid and liquid phases of sediment may be useful for establishing effect concentrations.

13.3.3 Toxic units can be used to help interpret the response of organisms to multiple chemicals in sediment. A toxic unit is the concentration of a chemical divided by an effect concentration. For example, a toxic unit of exposure can be calculated by dividing the measured concentration of a chemical in pore water by the water-only LC50 for the same chemical (Ankley et al. 1991a, (**197**)). Toxic units could also be calculated by dividing the concentration in a whole sediment sample by a threshold concentration in whole sediment (Kemble et al. 1994, Long and Morgan 1991, (**81**, **95**)). Toxicity expressed as toxic units may be summed and this may provide information on the toxicity of chemical mixtures (Ankley et al. 1991a,(**197**)).

13.3.4 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 (Burton and Ingersoll 1994, (**198**). Surveys of sediment toxicity are usually part of more comprehensive analyses of biological, chemical, geological, and hydrographic data. Statistical correlation can be improved and

costs reduced if subsamples are taken simultaneously for sediment tests, chemical analyses, and benthic community structure.

13.3.5 Descriptive methods such as toxicity tests with field-collected sediment should not be used alone to evaluate sediment contamination. An integration of several methods using the weight of evidence is needed to assess the effects of contaminants associated with sediment (Long et al. 1990; Ingersoll et al.1996; Ingersoll et al. 1997; MacDonald et al. 1996, (**199**, **97**, **98**, **96**)). 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 (Burton 1991, (**68**), Chapman et al. 1992, 1997, (**99**, **100**); Canfield et al.1994, 1996, 1998, (**200**, **118**, **201**).

13.3.6 Toxicity Identification Evaluation (TIE) procedures can be used to help provide insights as to specific contaminants responsible for toxicity in sediment (Ankley and Thomas 1992 (**57**), Ankley et al. 1991a,(**197**)). For example, the toxicity of contaminants such as metals, ammonia, hydrogen sulfide, and nonionic organic compounds can be identified using TIE procedures.

14. Report

14.1 The record of the results of an acceptable sediment test should include the following information either directly or by referencing available documents:

14.1.1 Name of test and investigator(s), name and location of laboratory, and dates of start and end of test.

14.1.2 Source of control or test sediment, method for collection, handling, shipping, storage, and disposal of sediment.

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

14.1.4 Source and characteristics of overlying water, description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

14.1.5 Source, history, and age of test organisms; source, history, and age of brood stock, culture procedures; and source and date of collection of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, age or life stage, means and ranges of weight or length, observed diseases or unusual appearance, treatments, holding, and acclimation procedures.

14.1.6 Source and composition of food, concentrations of test material and other contaminants, procedure used to prepare food, feeding methods, frequency, and ration.

14.1.7 Description of the experimental design and test chambers, the depth and volume of sediment and overlying water in the chambers, lighting, number of test chambers and number of test organisms/treatment, date and time test starts and ends, temperature measurements, dissolved oxygen concentration (as percent saturation), and any aeration used before starting a test and during the conduct of a test.

14.1.8 Methods used for physical and chemical characterization of sediment.

14.1.9 Definition(s) of the effects used to calculate LC50 or EC50s, biological endpoints for tests, and a summary of general observations of other effects.

14.1.10 Methods used for statistical analyses of data: *(1)* summary statistics of the transformed or raw data as applicable (for example, mean, standard deviation, coefficient of variation, precision and bias); *(2)* hypothesis testing (raw data, transformed data, null hypothesis, alternate hypothesis, target Type I and II error rates, statistics used (including calculation of test statistic)), decision rule used (for example, *W* statistic >0.65 results in the rejection of the null hypothesis), calculated test statistic and decision rule result, achieved Type I and II error rates (for some discrete tests, achieved error rates only approximate the target rates); *(3)* results of regression analyses (parameters of regression fit, uncertainty limits on the regression parameters, correlation coefficient).

14.1.11 Summary of general observations on other effects or symptoms.

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

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

15. Precision and Bias

15.1 *Determining Precision and Bias:*

15.1.1 Precision is a term that describes the degree to which data generated from replicate measurements differ and reflects the closeness of agreement between randomly selected test results. Bias is the difference between the value of the measured data and the true value and is the closeness of agreement between an observed value and an accepted reference value (Practices E177 and E691). Quantitative determination of precision and bias in sediment testing of aquatic organisms is difficult or may be impossible in some cases, as compared to analytical (chemical) determinations. This is due, in part, to the many unknown variables which affect organism response. Determining the bias of a sediment test using field samples is not possible since the true values are not known. Since there is no acceptable reference material suitable for determining the bias of sediment tests, bias of the procedures described in this standard has not been determined (section 15.2).

15.1.2 Sediment tests exhibit variability due to several factors. Test variability can be described in terms of two types of precision, either single laboratory (intralaboratory or repeatability; section 15.6.1) precision or multilaboratory (interlaboratory or reproducibility; sections 15.5.2 and 15.6) precision (also referred to as round-robin or ring tests). Intralaboratory precision reflects the ability of trained laboratory personnel to obtain consistent results repeatedly when performing the same test on the same organism using the same toxicant. Interlaboratory precision is a measure of how reproducible a method is when conducted by a large number of laboratories using the same method, organism, and toxic sample. Generally, intralaboratory results are less variable than interlaboratory results

(USEPA 1993a, Swartz 1989, USEPA 1993b, Marcus and Holtzman 1988, Grothe and Kimerle 1985, Pittinger et al. 1989, (**131, 104, 165, 202, 203, 204**)).

15.1.3 A measure of precision can be calculated using the mean and relative standard deviation (percent coefficient of variation, or CV % = standard deviation/mean × 100) of the calculated endpoints from the replicated endpoints of a test. However, precision reported as the CV should not be the only approach used for evaluating precision of tests and should not be used for the NOEC effect levels derived from statistical analyses of hypothesis testing. The CVs may be very high when testing extremely toxic or nontoxic samples. For example, if there are multiple replicates with no survival and one with low survival the CV may exceed 100 %, yet the range of response is actually quite consistent. Therefore, additional estimates of precision should be used, such as range of responses and minimum detectable differences (MDD) compared to control survival or growth. Several factors can affect the precision of the test, including test organism age, condition, sensitivity, handling, and feeding of the test organisms, overlying water quality, and the experience in conducting tests. For these reasons, it is recommended that trained laboratory personnel conduct the tests in accordance with the procedures outlined in Annex A1 and in Annex A2. Quality assurance practices should include: *(1)* single laboratory precision determinations that are used to evaluate the ability of the laboratory personnel to obtain precise results using reference toxicants for each of the test organisms and *(2)* preparation of control charts (Figure 16 in Test Method E1706) for each reference toxicant and test organism. The single laboratory precision determinations should be made before conducting a sediment test and should be periodically performed as long as whole-sediment tests are being conducted at the laboratory.

15.1.4 Intralaboratory precision data are routinely calculated for test organisms using water-only 96-h exposures to a reference toxicant such as Cd Cl_2. Intralaboratory precision data should be tracked using a control chart. Each laboratory's reference-toxicant data will reflect conditions unique to that facility, including dilution water, culturing, and other variables (Section 11). However, each laboratory's reference toxicant CVs should reflect good repeatability.

15.1.5 Two interlaboratory precision (round-robin) tests have been completed using 10-d whole sediment tests, one with *Rhepoxynius abronius* (Mearns et al. 1986 (**205**), and the other with *Ampelisca abdita*, *Eohaustorius estuarius*, and *Leptocheirus plumulosus* (Schlekat et al. 1995 (**22**)). The results of these round-robin studies are described in section 15.5.

15.1.6 One interlaboratory precision test has been completed on the 28-d chronic test with *Leptocheirus plumulosus* (DeWitt et al., 1997b (**26**). Ten laboratories participated in the round-robin study, which used a dilution series of highly contaminated Black Rock Harbor sediment from a Superfund site in Connecticut mixed with uncontaminated, diluent sediment from Sequim Bay, Washington. The results of this round-robin study are described in section 15.6.

15.2 *Bias*—The bias of toxicity tests cannot be determined since there is no acceptable reference material. The bias of the reference-toxicity tests can only be evaluated by comparing test responses to control charts.

15.3 *Replication and Test Sensitivity*—The sensitivity of sediment tests will depend in part on the number of replicates per concentration, the probability levels (alpha and beta), and the type of statistical analysis. For a specific level of variability, the sensitivity of the 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 (Section 13).

15.4 *Demonstrating Acceptable Laboratory Performance:*

15.4.1 Intralaboratory precision, expressed as a coefficient of variation (CV), 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 consistently provide partial mortalities at two or more concentrations of the test chemical (section 11.14 and Table 4). See section 11.16 for additional detail regarding reference-toxicity testing.

15.4.2 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 (that is, water quality) result in acceptable performance in the tests as outlined in Annex A1 and Annex A2.

15.4.3 A control chart can be prepared for each combination of reference toxicant and test organism. Each control chart should include the most current data. Endpoints from five tests are adequate for establishing the control charts. In this technique, a running plot is maintained for the values (X_i) from successive tests with a given reference toxicant (See Figure 16 in Test Method E1706), and the endpoint (LC50, NOEC, ICp) are examined to determine if they are within prescribed limits. Control charts as described in USEPA 1993a, 1993b, (**131, 165**) are used to evaluate the cumulative trend of results from a series of samples. The mean and upper and lower control limits (± 2 SD) are recalculated with each successive test result.

15.4.4 The outliers, which are values falling outside the upper and lower control limits, and trends of increasing or decreasing sensitivity, are readily identified using control charts. With an alpha of 0.05, one in 20 tests would be expected to fall outside of the control limits by chance alone. If 2 of 20 reference-toxicity tests fall outside the control limits, the sediment toxicity tests conducted during the time in which the second reference-toxicity test failed are suspect, and should be considered as provisional and subject to careful review.

15.4.5 A sediment test may be acceptable if specified conditions of a reference-toxicity test fall outside the expected ranges (section 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

TABLE 6 Inter-laboratory Precision for Survival of *Rhepoxynius abronius* in 10-d Whole Sediment Toxicity Tests Using Seven Sediments (Mearns et al. 1986 (205, USEPA 1994a (1))

	Percent Survival (SD) in Sediment Samples						
Lab	Control	4 mg/kg Cd	8 mg/kg Cd	12 mg/kg Cd	Central Basin	Sinclair Inlet	City Waterway
1	92 (7)	89 (7)	87 (9)	8 (3)	83 (11.5)	78 (13)	74 (11.5)
2	96 (4)	98 (3)	90 (10)	41 (11)	69 (7.5)	67 (11)	87 (12)
3	100 (0)	97 (3)	78 (10.5)	12 (7.5)	90 (8)	87 (7.5)	83 (12.5)
4	94 (7)	99 (2)	50 (15)	6 (5.5)	92 (5.5)	88 (3)	84 (11)
5	100 (0)	98 (4.5)	77 (3)	28 (11.5)	80 (3.5)	74 (9)	87 (3)
Mean	96.4 (3.6)	96.2 (4.1)	76.4 (15.8)	19 (15.5)	82.8 (9.1)	78.8 (8.9)	83 (5.3)
CV (%)	3.7	4.2	20.7	79.1	11.0	11.3	6.4

survival in the reference-toxicity test is <90 %. All the performance criteria outlined in Annex A1 and Annex A2 should 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.

15.4.6 If the value from a given test with the reference toxicant falls more than two standard deviation (SD) outside the expected range, the sensitivity of the organisms and the overall credibility of the test system may be suspect USEPA 1993a,(**131**). In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

15.4.7 Performance should improve with experience, and the control limits for point estimates should gradually narrow. However, control limits of ± 2 SD, by definition, will be exceeded 5 % of the time, regardless of how well a laboratory performs. Highly proficient laboratories which develop a very narrow control limit may be unfairly penalized if a test which falls just outside the control limits is rejected *de facto*. For this reason, the width of the control limits should be considered in determining whether or not an outlier is to be rejected. This determination should may be made by the regulatory authority evaluating the data.

15.4.8 The recommended reference-toxicity test consists of a control and five or more concentrations in which the endpoint is an estimate of the toxicant concentration which is lethal to 50 % of the test organisms in the time period prescribed by the test. The LC50 is determined by an appropriate procedure, such as the trimmed Spearman-Karber Method, Probit Method, Graphical Method, or the Linear Interpolation Method (Section 13 and Test Method E1706).

15.4.9 The point estimation analysis methods recommended in this test method have been chosen primarily because they are well-tested, well-documented, and are applicable to most types of test data. Many other methods were considered in the selection process, and it is recognized that the methods selected are not the only possible methods of analysis for toxicity data.

15.5 *Precision of 10-d Amphipod Sediment Toxicity Test Methods:*

15.5.1 *Intralaboratory Precision*—Intralaboratory precision has not been evaluated for any of the four amphipod species described in Annex A1.

15.5.2 *Interlaboratory Precision:*

15.5.2.1 Interlaboratory precision for *R. abronius* using 10-d whole sediment toxicity tests using the methods described in this standard (Table A1.1 in Annex A1) is described by Mearns et al. 1986 (**205**). Five laboratories participated in the study, including federal and state government laboratories, a contract laboratory, and an academic laboratory. The laboratories were chosen because each had demonstrated experience in sediment toxicity tests with *R. abronius*. The experimental design required each laboratory to conduct 10-d whole sediment tests on a total of 7 sediment treatments. One control sediment was tested. Three sediment treatments consisted of control sediment that was amended with $CdCl_2$ to result in the following measured concentrations: 4, 8, and 12 mg Cd/kg dry weight. Three field-collected sediments were also used. They were collected from the following locations in Puget Sound, WA: Central Basin (Metro Seattle Station A600E), inner Sinclair Inlet, and Slip No. 1 in City Waterway, Commencement Bay.

15.5.2.2 Amphipods were collected from a depth of 6 m off West Beach, Whidbey Island, WA, and distributed to each participating laboratory. Each laboratory used its own source of clean seawater.

15.5.2.3 All five laboratories had >90 % survival in control sediment, and thereby met the performance criteria for the test. Mean survival in control sediment was 96.4 %, the CV was 3.7 %, and the range in mean survival was from 92 to 100 % (Table 6). Of the cadmium-spiked sediments, survival was the least variable in the 4 mg/kg Cd treatment. Mean survival was 96.2 %, the CV was 4.2 %, and the range was from 89 to 98 %. The most variable response was in the 12 mg/kg Cd sediment. Mean survival was 19 %, the CV was 79.1 %, and the range was from 6 to 41 %. City Waterway showed the least variability among the field-collected sediments, with a mean survival of 83 %, a CV of 6.4 %, and a range from 74 to 87 %. Sinclair Inlet showed the greatest variability among the field-collected sediments, with a mean survival of 78.8 %, a CV of 11.3 %, and a range from 67 to 88 %.

15.5.2.4 Interlaboratory precision for *A. abdita*, *E. estuarius*, and *L. plumulosus* using 10-d whole sediment toxicity tests is described in Schlekat et al. (1995 (**22**)). Details of this study are described below. The number of participating laboratories varied with the test species: six for *A. abdita*, eight for *E. estuarius*, and seven for *L. plumulosus*. Laboratories were chosen on the basis of demonstrated experience with the particular test species. Each laboratory conducted 10-d sediment toxicity tests on 4 sediment treatments. Sediment treatments were selected for each species to include one negative control sediment and three contaminated sediments. Highly

TABLE 7 Inter-laboratory Precision for Survival of *Ampelisca abdita* in 10-d Whole Sediment Toxicity Tests Using Four Sediments (USEPA 1994a (1))

	Percent Survival (SD) in Sediment Samples			
Lab	Control	7 % Black Rock Harbor	20 % Black Rock Harbor	33 % Black Rock Harbor
1	97.0 (4.5)	63.0 (19.6)	10.0 (7.9)	6.0 (4.2)
2	94.0 (8.9)	75.0 (6.1)	7.0 (4.5)	0.0 (0)
3	97.0 (4.5)	90.0 (3.5)	36.0 (9.6)	38.0 (14.4)
4	94.0 (8.9)	79.0 (17.8)	7.0 (4.5)	3.0 (6.7)
5	85.0 (7.1)	20.0 (12.7)	1.0 (2.2)	1.0 (2.2)
6	100.0 (0)	97.0 (4.5)	90.0 (5.0)	72.0 (13.0)
Mean	94.5 (5.2)	70.7 (13.0)	25.2 (34.0)	20.0 (29.2)
CV (%)	5.5	38.9	135.1	146.2

TABLE 8 Interlaboratory Precision for Survival of *Eohaustorius estuarius* in 10-d Whole Sediment Toxicity Tests Using Four Sediments (USEPA 1994a (1))

	Percent Survival (SD) in Sediment Samples			
Lab	Control	9 % Black Rock Harbor	25 % Black Rock Harbor	42 % Black Rock Harbor
1	96.0 (6.5)	45.0 (19.7)	6.0 (6.5)	16.0 (9.6)
2	98.0 (2.7)	76.0 (10.8)	46.0 (13.9)	25.0 (7.1)
3	97.0 (2.7)	89.0 (4.2)	59.0 (10.8)	45.0 (10.0)
4	98.8 (2.7)	59.0 (23.0)	47.2 (23.2)	45.8 (27.0)
5	100.0 (0)	75.0 (19.7)	36.0 (12.4)	16.0 (9.6)
6	100.0 (0)	69.0 (12.9)	56.0 (18.8)	38.0 (14.4)
7	99.0 (2.2)	79.0 (6.5)	61.0 (10.8)	50.0 (7.9)
8	97.0 (6.7)	53.0 (14.4)	24.0 (14.7)	29.0 (15.6)
Mean	98.2 (1.5)	68.1 (14.7)	41.9 (19.1)	33.1 (13.5)
CV (%)	1.5	21.6	45.5	40.9

TABLE 9 Interlaboratory Precision for Survival of *Leptocheirus plumulosus* in 10-d Whole Sediment Toxicity Tests Using Four Sediments (USEPA 1994a (1))

	Percent Survival (SD) in Sediment Samples			
Lab	Control	10 % Black Rock Harbor	28 % Black Rock Harbor	47 % Black Rock Harbor
1	91.3 (4.8)	6.0 (4.2)	5.0 (3.5)	2.5 (2.9)
2	91.0 (8.9)	62.0 (11.0)	51.0 (15.6)	33.0 (11.5)
3	88.0 (8.4)	34.0 (15.2)	22.0 (13.0)	7.0 (5.7)
4	92.0 (7.6)	48.0 (23.9)	59.0 (21.6)	27.0 (10.4)
5	86.0 (10.2)	20.0 (9.4)	28.0 (4.5)	12.0 (9.1)
6	95.0 (6.1)	76.0 (10.2)	65.0 (14.6)	38.0 (17.5)
7	99.0 (2.2)	78.0 (13.0)	56.0 (4.2)	26.0 (6.5)
Mean	91.8 (4.3)	46.3 (27.7)	40.9 (22.6)	20.8 (13.6)
CV (%)	4.7	59.8	55.2	65.5

contaminated sediment from Black Rock Harbor, CT, was diluted with species-specific, non-contaminated control sediment, creating test sediments that ranged in relative contamination from low to high.

15.5.2.5 Independent suppliers distributed amphipods to each laboratory. *Ampelisca abdita* and *E. estuarius* were field-collected from locations in Narragansett, RI, and Newport, OR, respectively. *Leptocheirus plumulosus* were obtained from cultures located at the University of Maryland, Queenstown, MD. Each laboratory used its own supply of clean seawater.

15.5.2.6 Mean survival of *A. abdita* in control sediment ranged from 85 to 100 % (Table 7). Five of the six laboratories achieved greater than 90 % survival in control sediment, which is the minimum survival that must be obtained in control sediment in order for the test to be accepted. The grand mean was 94.5 %, and the CV was 5.5. A dose response was exhibited with decreasing survival with increasing proportions of BRH sediment. Test sediments (that is, 7, 25, and 33 % BRH dilutions) exhibited a higher degree of variability than in control sediment. In 7 % BRH sediment, mean survival ranged from 20 % in Laboratory 5 to 97 % in Laboratory 6 (Table 7). Twenty- percent BRH exhibited the greatest magnitude of variability, with a range of 1 to 90 %. Thirty-three percent BRH also exhibited considerable variability. The overall rank of sediment toxicity as measured by absolute mortality was consistent among laboratories. One hundred percent of laboratories were in agreement for in ranking control and 7 % BRH sediments as the first and second least toxic sediments, respectively (Table 7).

15.5.2.7 Every laboratory surpassed the minimum survival criteria of 90 % survival in control sediment with *E. estuarius*. The range was from 96 to 100 %, with a grand mean of 98.2 % and a CV of 1.5 (Table 8). Grand mean survival decreased with increasing proportions of BRH. BRH sediment dilutions exhibited greater variability than control sediment, with 25 % BRH displaying the highest coefficient of variation. All eight laboratories ranked survival of *E. estuarius* for control and 9 % BRH as the least and second least toxic, respectively (Table 7). With the exception of Laboratories 1 and 8, the rank for 25 and 42 % BRH were appropriately third and fourth least toxic, respectively.

15.5.2.8 *Leptocheirus plumulosus* exhibited a range of survival in control sediment from 86 to 99 % (Table 9). The grand mean was 91.8 %, and the CV was 4.7. Two laboratories, 3 and 5, failed to meet the minimum control sediment survival criteria of 90 %. Grand means displayed a dose response of decreasing survival with increasing proportion of BRH sediment. Coefficients of variation were uniformly higher in BRH sediment dilutions as compared to control sediment, but did not vary greatly among BRH sediments (Table 9). Laboratory 1 appeared to be an outlier with respect to survival in BRH sediment dilutions, as survival of *L. plumulosus* was the lowest for all three BRH sediments for any laboratory. The rank of sediments according to their toxicity was generally consistent among laboratories. Agreement was 100 % for control and the highest BRH sediment; these were appropriately ranked 1and 4, respectively (Table 9). Laboratories 4 and 5 anomalously ranked 10 and 28 % BRH as 3 and 2, respectively, whereas the remaining laboratories ranked these sediments appropriately according to the proportion of BRH.

15.5.3 These tests exhibited similar or better precision than many chemical analyses and effluent toxicity testing methods (USEPA, 1991 (**126**)). The success rate for test initiation and completion of this round-robin evaluation is a good indication that a well equipped and trained staff will be able to successfully conduct this test. This is an important consideration for any test performed routinely in any regulatory program.

15.6 *Precision of the 28-d Sediment Toxicity Test Methods with Leptocheirus plumulosus:*

15.6.1 *Intralaboratory Performance*—Studies described in DeWitt et al. (1997b (**26**)) provide data that can be used to characterize intralaboratory precision with the 28-day long-term toxicity test with *L. plumulosus*. These data provide an

TABLE 10 Intralaboratory Precision Distribution of the Coefficient of Variation for Each Test Endpoint (DeWitt et al. 1997a; USEPA-USACE 2001 (2))

Endpoint	Sample Size	Mean	Median	Minimum	Maximum	1st Quartile	3rd Quartile
% Survival (Arcsine transformed)	88	14 %	11 %	0 %	173 %	8 %	14 %
Growth rate (log transformed)	87	4 %	3 %	0 %	16 %	2 %	6 %
Reproduction (square root transformed)	88	31 %	18 %	0 %	141 %	13 %	36 %

estimate of intralaboratory precision from a single laboratory from a total of 88 treatments (Table 10). To be consistent with standard statistical procedures, these data were transformed to reduce the heterogeneity of within class variance. Percent survival was transformed to the arcsine-square root of the value; growth rate was transformed to the natural logarithm of the value; and reproduction (offspring per survivor) was transformed to the arcsine -square root of the value. A CV was calculated on the transformed data for each treatment within an experiment. The observed distribution obtained from the resulting sample of CVs from all experiments was then characterized. This distribution of CVs then provides an appropriate range on which to base sample size calculations for future experiments. The median CVs were 11 % for survival, 3 % for growth rate, and 18 % for reproduction (Table 10). The range between the first and third quartiles provides a useful nonparametric interval bounding the distribution. This range was 8 to 14 % for survival, 2 to 6 % for growth rate, and 13 to 36 % for reproduction (Table 10). These values are similar to CVs for intralaboratory precision calculated for survival from 10-d tests with control sediment using *Hyalella azteca* and *Chironomus tentans* (7.2 % and 5.7 %, respectively; USEPA 2000 and Test Method E1706).

15.6.2 *Interlaboratory Precision:*

15.6.2.1 Interlaboratory precision for *L. plumulosus* in the 28-d whole sediment toxicity test using methods similar to those described in this standard (Table A2.1 in Annex A2) was evaluated by round-robin testing (DeWitt et al., 1997b (**26**). Ten laboratories, including federal and state government laboratories, contract laboratories, and academic laboratories with demonstrated experience in chronic toxicity testing using *L. plumulosus*, participated in round-robin toxicity testing (DeWitt et al., 1997b (**26**). The experimental design required each laboratory to conduct the 28-d chronic test using a dilution series of Black Rock Harbor sediment (BRH; a Superfund site in Connecticut) mixed with clean, diluent sediment from Sequim Bay, Washington. Each sediment treatment was prepared in a single batch that was subsampled and shipped to testing laboratories. A total of four concentrations of BRH sediment and one negative control sediment were tested. Across all treatments, total organic carbon averaged 2.6 % dry weight, total solids averaged 33 %, and grain size averaged 15 % sand, 42 % silt, and 43 % clay. In general, cadmium, chromium, copper, lead, nickel, and zinc, as well as total PAHs, increased along the dilution series gradient. Table 11 summarizes the concentration ranges for the inorganic contaminants.

15.6.2.2 About 4 months before the start of the round-robin study, laboratories not currently maintaining cultures of *L. plumulosus* were supplied with amphipods, sediment, food, and culturing methods by the Battelle Marine Sciences Laboratory (MSL). Each laboratory maintained cultures following the culturing method detailed in DeWitt et al. (1997a (**7**)). Each laboratory used its own source of clean seawater.

TABLE 11 Ranges of the BRH Sediment Dilution Series Chemical Concentrations (mg/kg dry wt; from DeWitt et al., 1997b(26 and USEPA-USACE 2001 (2))

	Low (BRH treatment)	High (BRH treatment)
Cadmium	4.09 (0.0 %)	13.5 (15.1 %)
Chromium	104 (0.0 %)	767 (15.1 %)
Copper	104 (0.0 %)	1503 (15.1 %)
Lead	31.1 (0.0 %)	209 (15.1 %)
Nickel	91.2 (0.0 %)	150 (15.1 %)
Zinc	189 (0.0 %)	736 (15.1 %)
Total PAHs	9.85 (1.4 %)	17.5 (15.1 %)

15.6.2.3 Of the ten laboratories participating in the round-robin, only five laboratories had >80 % survival in the negative control sediment, and thereby met this performance criterion for test acceptability (Top of Table 12). Analysis of the data resulting from the round-robin included only these five laboratories. Mean survival in the negative control sediment was 93.6 %, the CV was 4.2 %, and the range was from 89 to 98 % (Table 12). The CVs across laboratories from the five treatments ranged from 3.1 to 12.8 %, with a mean of 8.4 %, and increased with dose. None of the laboratories produced less than 70 % survival, even in the highest concentration of BRH sediment. Further, none of the laboratories produced a monotonic dose response for survival. This suggests that the test did not contain a wide enough series of dilutions to adequately measure the response of survival. For those laboratories that showed a statistically significant decrease in survival in the highest concentration of BRH (n=4), an average of 16 % change in survival was produced between the control and the highest concentration of BRH sediment.

15.6.2.4 For the five laboratories that met the performance criterion, interlaboratory precision for this study was characterized by the maximum and minimum CV for each endpoint. The minimum interlaboratory CV averaged about 4 % for survival, 14 % for growth rate, and 35 % for reproduction (Table 13). Maximum interlaboratory CV averaged 19 % for survival, 38 % for growth rate, and 102 % for reproduction. The interlaboratory MDD for survival ranged from 8 to 31 %, and the intralaboratory MDD for survival ranged from 10 to 26 %. The interlaboratory MDD for growth rate ranged from 0.011 to 0.017 mg/individual/d, and the intralaboratory MDD for growth rate ranged from 0.009 to 0.024 mg/individual/d. The interlaboratory MDD for reproduction ranged from 0.33 to 2.86 offspring per survivor, and the intralaboratory MDD for reproduction ranged from 0.92 to 2.73 offspring per survivor. These MDDs should be interpreted cautiously, because they are derived from one study consisting of a small number of comparisons. Although the technical staff for laboratories

TABLE 12 Results of Round-robin Interlaboratory Precision of Endpoint Sensitivity for *L. plumulosus* in a 28-d Long-term Toxicity Test Using Black Rock Harbor Sediments (DeWitt et al., 1997b (26 and USEPA-USACE 2001 (2))

A) Results for Laboratories that met Control Performance Criteria Concentration of Black Rock Harbor Sediment						
Lab	0.0 %	1.4 %	4.6 %	8.3 %	15.1 %	
			Mean Percent Survival (%CV)			MDD %
4	89 (11.5)	92 (3.0)	82 (17.6)	76 (16.4)	73 (13.4)	16
6	96 (6.8)	93 (2.9)	97 (4.6)	95 (7.4)	96 (5.7)	8
7	90 (6.8)	88 (9.5)	84 (12.9)	92 (6.2)	82 (11.9)	13
8	95 (6.4)	92 (6.2)	72 (42.4)	74 (42.0)	70 (18.2)	31
9	98 (2.8)	96 (2.3)	84 (15.4)	91 (10.6)	86 (14.5)	14
Mean	93.6	92.2	83.8	85.6	81.4	
%CV	4.2	3.1	10.6	11.5	12.8	
MDD %	10	7	26	24	16	
			Mean Growth Rate mg/d (%CV)			MDD mg/ind/d
6	0.059 (9.8)	0.054 (6.0)	0.046 (19.0)	0.039 (11.7)	0.020 (24.1)	0.009
7	0.084 (4.4)	0.075 (4.9)	0.063 (8.5)	0.053 (7.2)	0.035 (28.0)	0.009
8	0.045 (18.3)	0.031 (12.7)	0.036 (25.1)	0.024 (27.5)	0.014 (14.1)	0.010
9	0.089 (8.7)	0.078 (13.4)	0.065 (12.7)	0.060 (12.0)	0.045 (11.6)	0.012
Mean	0.063	0.057	0.049	0.039	0.025	
%CV	35.8	35.7	29.8	45.1	59.4	
MDD	0.014	0.014	0.017	0.012	0.011	
			Mean Offspring per Survivor (%CV)			MDD # offspring
4	0.27 (141)	2.26 (72.3)	0.65 (149)	0.35 (56.5)	0.33 (81.2)	1.33
6	4.37 (41.0)	2.96 (53.8)	2.58 (27.5)	1.70 (43.4)	0.18 (76.6)	1.77
7	5.22 (55.7)	3.99 (40.5)	3.61 (42.5)	2.21 (75.4)	0.48 (65.6)	2.73
8	1.66 (65.8)	1.10 (54.2)	1.52 (29.8)	0.25 (91.5)	0.10 (108)	0.92
9	7.09 (30.8)	5.43 (21.9)	3.48 (29.8)	1.65 (60.7)	0.19 (99.0)	1.96
Mean	3.72	3.15	2.37	1.23	0.25	
%CV	73.8	52.5	53.8	71.2	59.5	
MDD	2.86	2.10	1.53	1.42	0.33	
B) Results for Laboratories that did not meet the Control Performance Criteria Concentration of Black Rock Harbor Sediment						
Lab	0.0 %	1.4 %	4.6 %	8.3 %	15.1 %	
			Mean Percent Survival (%CV)			
1	53 (31.7)	74 (13.0)	65 (38.5)	58 (18.9)	39 (64.4)	
2	0 (-)	10 (-)	27 (137.1)	15 (-)	0 (-)	
3	72 (34.6)	85 (17.1)	74 (15.4)	61 (21.2)	55 (24.9)	
5	60 (56.5)	88 (18.7)	66 (29.5)	84 (24.7)	76 (11.8)	
10	69 (29.6)	59 (49.9)	58 (44.2)	37 (70.0)	25 (58.3)	
			Mean Growth Rate mg/ind/d (%CV)			
1	0.024 (81.7)	0.032 (37.7)	0.012 (74.9)	0.012 (67.9)	0.008 (71.2)	
2	0 (-)	0.027 (-)	0.028 (49.0)	0.017 (-)	0 (-)	
3	0.050 (50.2)	0.067 (21.0)	0.055(33.3)	0.034(52.4)	0.025 (32.0)	
5	0.058 (16.0)	0.062 (31.7)	0.037 (67.6)	0.036 (43.0)	0.024 (12.0)	
10	0.006 (54.5)	0.014 (139)	0.007 (47.1)	0.003 (54.0)	0.003 (80.2)	
			Mean Offspring per Survivor (%CV)			
1	0.7 (45.2)	1.7 (57.0)	0.4 (206)	0.1 (163)	0 (-)	
2	0 (-)	1.3 (-)	1.2 (18.0)	0.6 (-)	0 (-)	
3	4.8 (42.5)	3.7 (51.5)	3.4 (34.9)	0.4 (92.4)	0 (138)	
5	3.1 (80.8)	2.3 (25.5)	1.1 (136)	0.8 (113)	0.6 (117)	
10	0.1 (131)	1.4 (111)	0.5 (98.3)	0.8 (157)	0.3 (144)	

TABLE 13 Summary of Interlaboratory Precision at Five Laboratories for the 28-Day *Leptocheirus plumulosus* Chronic Test Using Five Dilutions of Black Rock Harbor Sediment (DeWitt et al. 1997b(26 and USEPA-USACE 2001(2))

	Lab-4	Lab-6	Lab-7	Lab-8	Lab-9
Survival					
Min CV (%)	3	3	6	6	2
Max CV (%)	18	7	13	42	15
Growth rate					
Min CV (%)	36	6	4	13	9
Max CV (%)	96	24	28	27	13
Offspring per Survivor					
Min CV (%)	56	27	40	30	22
Max CV (%)	149	77	75	108	99

participating in the round-robin had extensive sediment toxicity testing experience, many had limited testing experience specifically with *L. plumulosus*. Therefore, these values for interlaboratory precision may be higher than would be expected from laboratories with routine experience testing with this species.

15.6.2.5 A cost-power analysis was conducted on round-robin data to determine the number of replicates required per treatment for the 28-d whole-sediment standard testing using *L. plumulosus* (DeWitt et al., 1997b (**26**). This analysis involved evaluating both the improvement in statistical power of the test to detect a difference between treatment means and the additional expense of adding more replicates. For this analysis, the cost of a replicate was assumed to be proportionate to the time required to conduct all of the tasks associated with one treatment. If cost was not a concern, 14 replicates would be optimal and would provide 80 % power for detecting a 30 % difference in reproduction at a CV of about 36 %. This number of replicates is impractical because of costs and logistics. The cost-power analysis for the *L. plumulosus* chronic test indicated that six replicates per treatment gives the greatest statistical power at the most efficient cost. However, this conclusion was based on the assumption that every 1 % increase in improved detection equals a 1 % increase in cost. The decision to specify 5 replicates per treatment in this standard was based primarily on an effort to keep the cost of performing this test to a minimum. Based on the median CVs for growth rate, survival, and reproduction calculated from a large data set (3 %, 11 %, and 18 %, respectively), five replicates will provide high power (>0.80) to detect a 20 % decrease in survival and growth rate endpoints relative to the control (see Figure 12.5 in USEPA-USACE 2001 (**2**)). For the reproduction endpoint, the power to detect a 20 % decrease will be closer to 0.40 using five replicates and 0.50 using six replicates. With power fixed at 80 % and at a CV of 20 %, the median CV demonstrated for reproduction with five replicates would be suitable to detect about 18 % reduction in reproduction and with six replicates about 16 % reduction. Thus, there is relatively little gained by increasing the number of replicates from five to six. Nevertheless, if reproduction is the assessment endpoint of most concern, then incorporation of more than five replicates should be considered. Because space and cost considerations make use of five replicates desirable, this method would benefit from additional research to find ways to reduce the among-replicate variability for the reproduction endpoint.

15.6.2.6 The mean growth rates across the laboratories for each dose decreased with increasing concentration of BRH sediment (Table 13). Thus, the growth rate was a more sensitive measure to the concentration of BRH survival. The CVs across the laboratories from the five treatments ranged from 29.8 to 59.4 %, with a mean of 41.2 %, and were on average five times greater for growth rate than for survival (Table 13). Of the five laboratories that met the performance criterion for control survival, three laboratories produced a monotonic dose response to growth rate. The percentage of change in the growth rate between control and the highest concentration of BRH sediment was on average 58 % for these three laboratories.

15.6.2.7 The mean reproduction across laboratories for each dose decreased with increasing concentration of BRH sediment. Thus, the measure of reproduction was more sensitive to the concentration of BRH than was survival; however, the CVs across laboratories are on average eight times greater for reproduction than for survival. The CVs for the five treatments ranged from 52.5 % to 73.8 %, with a mean of 62.2 %. Of the five laboratories that met the performance criteria for control survival, three laboratories produced a monotonic dose response in reproduction. The percentage of change in reproduction (offspring/survivor) between the control and the highest concentration of BRH sediment was on average 95 % for these three laboratories.

15.6.2.8 USEPA (2000 (**206**)) and Test Method E1706 included a review of a series of round-robin studies from which interlaboratory precision was analyzed. CVs for survival in 10-d whole-sediment tests with *H. azteca* ranged from 6 to 114 % in three test sediments. Similar tests with *C. tentans* produced CVs of 8 to 181 % in three test sediments. In 28-d whole-sediment tests with *H. azteca*, CVs from five test sediments ranged from 7 to 28 % for survival , from 52 to 78 % for growth (dry weight), and from 66 to 193 % for reproduction.

15.6.2.9 The *Leptocheirus* round-robin study exhibited similar or better intra- and interlaboratory precision than many chemical analyses and toxicity testing methods (for example, USEPA, 1991 (**126**)). The cause(s) of the high failure rate among laboratories participating in the round-robin study is not known. Several of the laboratories had not conducted this toxicity test previously, and inexperience with the procedures may have contributed to some of the test failures. Some of the laboratories suggested that uneaten food might have accumulated during early days of the experiment, which might have led to lethal low-DO stress to the young amphipods (DeWitt et al. 1997b (**26**). Because of this potential problem, additional experiments were conducted (Annex A2) to find the minimum food ration that would minimize the build-up of excess food, minimize mortality, and produce significant growth rate and reproduction endpoints in the 28-d *L. plumulosus* sediment toxicity test. The diet recommended in this standard (A2.3.6) is based on the results of that experiment.

16. Keywords

16.1 *Ampelisca abdita*; amphipod; bioavailability; chronic; *Eohaustorius estuarius*; estuarine; invertebrates; *Leptocheirus plumulosus*; marine; *Rhepoxynius abronius*; sediment; toxicity

ANNEXES

(Mandatory Information)

A1. PROCEDURE FOR CONDUCTING A 10-D SEDIMENT SURVIVAL TEST WITH THE AMPHIPODS *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius*

A1.1 Introduction

A1.1.1 *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, and *Rhepoxynius abronius* have been used extensively to test the toxicity of estuarine or marine sediments. The choice of these amphipod species as test organisms is based on sensitivity to sediment-associated contaminants, availability and ease of collection, tolerance of environmental conditions (for example, temperature, salinity, grain-size), ecological importance, and ease of handling in the laboratory. Additionally, the species chosen for this method are intimately associated with sediment by nature of their burrowing or tube-dwelling and feeding habits. Field validation studies have shown that amphipods are absent or have reduced abundances at sites where toxicity in laboratory tests. Amphipod sediment toxicity tests have been successfully performed for regulatory and research purposes by numerous laboratories, including state and federal government agencies, private corporations, and academic institutions. Test guidance for *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* has been described (USEPA 1994a (**1**), Environment Canada 1992 (**5**)). The four species chosen are representative of both estuarine and marine habitats and sediments that span the spectrum of particle sizes from fine-to coarse-grained sediment. Thus, either alone or in combination, they may be used to measure toxicity of any commonly encountered estuarine or marine sediments (See Section 1 for additional details).

A1.1.2 Specific test methods for conducting the 10-d sediment toxicity test for the amphipods *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, and *Rhepoxynius abronius* are described in section A1.2. This test method was developed based on Swartz et al. (1985 (**10**)); DeWitt et al. (1989 (**11**)); Scott and Redmond (1989 (**12**)); Schlekat et al. (1992 (**13**)); and Environment Canada (1992 (**5**)). Results of tests using procedures different from the procedures described in section A1.2 may not be comparable and 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 estuarine or marine organisms. If tests are conducted with procedures different from the procedures described in this standard, additional tests are required to determine comparability of results (sections 1.4 and 1.10).

A1.2 Recommended Test Method for Conducting a 10-d Sediment Toxicity Test with *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius*

A1.2.1 Recommended conditions for conducting a 10-d sediment toxicity test with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* are summarized in Table A1.1. A general activity schedule is outlined in Table A1.2. 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 (Section 13). The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. When variability remains constant, the sensitivity of a test increases as the number of replicates increase.

A1.2.2 The recommended 10-d sediment toxicity test with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius* is conducted at the species-specific temperature and salinity with a 24 h light photoperiod at a illuminance of about 500 to 1000 lux (Table A1.1). Test chambers are 1-L glass chambers containing 175 mL of sediment and 775 mL of overlying seawater. Twenty amphipods are added to each test chamber at the start of a test. The size range of the amphipods will depend on species that is being tested (see section 12.3.4 for allowable size range for each species). The number of replicates/treatment depends on the objective of the test. Five replicates are recommended for routine testing (Section 13). Exposure is static (that is, water is not renewed), and the animals are not fed over the 10-d exposure period. Overlying water can be culture 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 where sediment is collected. For all other applications, the characteristics of the overlying water for each species should be chosen according to Table A1.1. Requirements for test acceptability are summarized in Table A1.3.

A1.3 General Procedures

A1.3.1 *Sediment into Test Chambers:*

A1.3.1.1 On the day before the addition of amphipods (Day -1), each test sediment (either field collected or laboratory spiked) should be homogenized by stirring in the sediment storage container or by using a rolling mill, feed mixer, or other suitable apparatus. Control and reference sediments are included. Sediment should be visually inspected to judge the extent of homogeneity. 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.

A1.3.1.2 A 175-mL aliquot of thoroughly homogenized sediment is added to each test chamber. It is important that an identical volume be added to each replicate test chamber; the volume added should equate to a depth of 2 cm in the test chamber. The sediment added to the test chamber should be settled either by tapping the side of the test chamber against the side of the hand or by smoothing the sediment surface with a

TABLE A1.1 Test Conditions for Conducting a 10-d Sediment Toxicity Test with *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius* (USEPA 1994a (1))

Parameter	Conditions
1. Test type:	Whole sediment toxicity test, static.
2. Temperature:	15°C: *E. estuarius* and *R. abronius* 20°C: *A. abdita* 25°C: *L. plumulosus*
3. Salinity:	28 ‰ : *A. abdita* and *R. abronius* 20 ‰ : *E. estuarius* and *L. plumulosus* Alternatively, the salinity of the overlying water can be adjusted to the salinity of the pore water at the site of interest for tests with *E. estuarius* or *L. plumulosus*. If tests are conducted at a different salinity, additional tests are required to determine comparability of results (sections 1.4 and 1.10).
4. Light quality:	Wide-spectrum fluorescent lights
5. Illuminance:	500 to 1000 lux
6. Photoperiod:	24 light
7. Test chamber:	1-L glass beaker or jar with 10-cm inner diameter.
8. Sediment volume:	175 mL (about 2-cm depth)
9. Overlying water volume:	775 mL
10. Renewal of overlying water:	None
11. Size and life stage of amphipods:	*A. abdita*: 3 to 5 mm (no mature males or females) *E. estuarius*: 3 to 5 mm *L. plumulosus*: 2 to 4 mm (no mature males or females) *R. abronius*: 3 to 5 mm
12. Number of organisms/chamber:	20 per test chamber
13. Number of replicate chambers/treatment:	Depends on objectives of test. At a minimum, four replicates should be used.
14. Feeding:	None
15. Aeration:	Water in each test chamber should be aerated overnight before start of test, and throughout the test; aeration at rate that maintains >90 % saturation of dissolved oxygen concentration.
16. Overlying water:	Clean sea water, natural or reconstituted water.
17. Overlying water quality:	Temperature daily. pH, ammonia, salinity, and DO of overlying water at least at test start and end. Salinity, ammonia, and pH of pore water.
18. Test duration:	10 d
19. Endpoints:	Survival (reburial optional for *E. estuarius*, *L. plumulosus*, and *R. abronius*)
20. Test acceptability:	Minimum mean control survival of 90 % and satisfaction of performance-based criteria specifications outlined in Table A1.3.

TABLE A1.2 General Activity Schedule for Conducting a Sediment Toxicity Test with *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius* (USEPA 1994a (1))

Day	Activity
-10 to -3	Collect or receive amphipods from supplier and place into collection site sediment. Alternatively, separate 2 to 4 mm *L. plumulosus* from cultures.
-9 to -2	Acclimate and observe amphipods to species-specific test conditions. Feed *A. abdita* and *L. plumulosus*. Monitor water quality (for expample, temperature, salinity, and dissolved oxygen).
-1	Observe amphipods, monitor water quality. Add sediment to each test chamber, place chambers into exposure system, and start aeration.
0	Measure pore-water total ammonia, salinity, and pH. Measure temperature of overlying water in test chambers. Transfer 20 amphipods into each test chamber. Archive 20 test organisms for length determination.
1	Measure temperature. Observe behavior of test organisms and ensure that each test chamber is receiving air. Measure dissolved oxygen in test chambers to which aeration has been cut-off.
2	Measure total water quality (pH, temperature, dissolved oxygen, salinity, total ammonia of overlying water). Observe behavior of test organisms and ensure that each test chamber is receiving air.
3 to 7 and 9	Same as Day 1.
8	Same as Day 2.
10	Measure temperature. End the test by collecting the amphipods with a sieve.

nylon, fluorocarbon, or polyethylene spatula. Highly contaminated sediment should be added to test chambers in a certified laboratory fume hood.

A1.3.2 *Addition of Overlying Water*—As test water is added, disruption of the sediment surface should be minimized. One way to accomplish this is by use of a turbulence reducer. Possible designs of turbulence reducer include a disk cut from polyethylene, nylon, or polytetrafluoroethylene (PTFE) sheeting (4 to 6 mil), or a glass petri dish attached (open face up) to a glass pipette. If a disk is used as the turbulence reducer, it should fit the inside diameter of the test chamber and have attached a length of nylon monofilament (or nontoxic equivalent) line. The turbulence reducer is positioned just above the sediment surface and raised as sea water is added to the 750-mL mark on the side of the test chamber. The turbulence reducer is removed and rinsed with test sea water between replicates of a treatment. A separate turbulence reducer is used for each treatment The test chambers should be covered, placed in a temperature controlled water bath (or other acceptable equivalent) and gently aerated. A test begins when the organisms are added to the test chambers (Day 0).

A1.3.3 *Addition of Amphipods*—On the following day (Day 0), amphipods are added to the test chambers. About one-third more amphipods than are needed for the test should be sieved

TABLE A1.3 Test Acceptability Requirements for a 10-d Sediment Toxicity Test with *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius* (USEPA 1994a (1))

A. It is recommended for conducting a 10-d test with *A. abdita*, *E. estuarius*, *L. plumulosus*, or *R. abronius* that the following performance criteria are met: 1. Size, life stage, and reproductive stage of amphipods must be within the prescribed species-specific ranges at the end of the test (Section 12). 2. Average survival of amphipods in the control sediment must be greater than or equal to 90 % at the end of the test. 3. Salinity, pH, and ammonia in the overlying water and sediment grain size should be within tolerance limits of test species.
B. Performance-based criteria for culturing *L. plumulosus* include: 1. Laboratories should perform periodic 96-h water-only reference-toxicity tests (at a minimum, one test every six months) to assess the sensitivity of culture organisms (section 11.16). 2. Records should be kept on the frequency of restarting cultures. 3. Laboratories should record the pH and ammonia of the cultures at least quarterly. Dissolved oxygen and salinity should be measured weekly. Temperature should be recorded daily. 4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.
C. Performance-based criteria for field-collected amphipods include: 1. Laboratories should perform reference-toxicant tests on each batch of field-collected amphipods received used in a sediment test (section 11.16). 2. Acclimation rates to test salinity and temperature should not exceed 3°C and 5 ‰ per 24 h. 3. Amphipods used in a toxicity test should exhibit active swimming behavior upon placement in water, have full digestive tracts, and display an acceptable color.
D. 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 solvent control used must not adversely affect test organisms. 5. The time-weighted average of daily temperature readings must be within ±2°C of the desired temperature. The instantaneous temperature must always be within ±3°C of the desired temperature. 6. The time-weighted average of daily salinity readings should be within ± 2 ‰ of the selected salinity and the instantaneous salinity readings should be ± 3 ‰ of the selected salinity. 7. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.

from the culture or control sediment in the holding container(s), and transferred to a sorting tray. The additional animals allow for the selection of healthy, active individuals. The sieve size for isolating amphipods from the culture or control sediment will depend upon the selected species. *Ampelisca abdita* and *L. plumulosus* should be isolated using a 0.5-mm sieve, whereas *E. estuarius* and *R. abronius* should be isolated using a 1.0-mm sieve. Sieving should be conducted with sea water of the same temperature and salinity as the holding and test water. Once isolated, active amphipods should be impartially selected using a transfer pipette or other suitable tool (not forceps), and distributed among dishes or cups containing test sea water until each container has twenty amphipods. The number of amphipods in each dish should be verified by recounting before adding to test chambers. To facilitate recounting, amphipods may be distributed to test chambers in batches of 5 or 10 instead of the full complement of 20. The distribution of amphipods to the test chambers should be impartial.

A1.3.3.1 Amphipods should be added to test chambers without disruption of the sediment. Any amphipods remaining in the sorting container should be gently washed into the test chamber using test sea water. The water level should be brought up to the 950-mL mark, the test chamber covered, and aeration may be discontinued for up to 1 to 2 h to allow the amphipods to burrow into the sediment. Aeration should then continue for the remainder of the test.

A1.3.3.2 After the addition of the animals, the test chambers should be examined for animals that may have been injured or stressed during the isolation, counting, or addition processes. Injured or stressed animals will not burrow into sediments, and should be removed. The period of time allowed for healthy amphipods to bury into test sediments will depend upon the species used. *Eohaustorius estuarius*, *L. plumulosus*, and *R. abronius* should be allowed 5 to 10 min to bury into the test sediment. *Ampelisca abdita*, which may take longer to build tubes, should be allowed 1 h. Amphipods that have not burrowed within the prescribed time should be replaced with animals from the same sieved population, unless they are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response. In that case, the amphipods are not replaced. The number of amphipods that are removed should be recorded.

A1.3.4 *Test Conditions:*

A1.3.4.1 *Aeration*—The overlying sea water in each test chamber should be aerated continuously after the water is added (that is, Days -1 through 10) except during introduction of the test organisms. Compressed air, previously filtered and free of oil, should be bubbled through a glass or plastic pipette and attached plastic tubing. The tip of the pipette should be suspended 2 to 3 cm above the surface of the sediment layer so as to not disturb the sediment surface. The concentration of dissolved oxygen (DO) in the water overlying the sediment in the test chambers is maintained at or near saturation by gently aerating the water. Air is bubbled through the test chamber at a rate that maintains a >90 % DO concentration, but does not cause turbulence or disturb the sediment surface. If air flow to one or more test chambers is interrupted for more than one hour, DO should be measured in those test chambers to determine whether DO concentrations have fallen below 60 % of saturation. Results may be unacceptable for test chambers in which aeration was interrupted and DO concentrations fell to below 60 % saturation.

A1.3.4.2 *Lighting*—Lights should be left on continuously at an intensity of 500 to 1000 lux during the 10-d exposure period. The constant light increases the tendency of the organisms to remain buried in the sediment, and thus to remain exposed to the test material.

A1.3.4.3 *Feeding*—The four species of amphipods used in this method are not be fed during the 10-d exposure period.

A1.3.4.4 *Water Temperature*—The test temperature will depend on the species that is tested. Test temperatures were selected to be near the summertime thermal maximum that

TABLE A1.4 Limits on Environmental Conditions Under Which to Conduct the 10-d Sediment Toxicity Tests with *Ampelisca abdita*, *Eohaustorius estuarius*, *Leptocheirus plumulosus*, or *Rhepoxynius abronius* (USEPA 1994a (1))

Parameter	*Ampelisca abdita*	*Eohaustorius estuarius*	*Leptocheirus plumulosus*	*Rhepoxynius abronius*
Temperature (°C)	20	15	25	15
Overlying water salinity (‰)	>10	1 to35	1.5 to 32	>25
Grain size (% silt/clay)	>10	<70 clay[A]	Full range	<90
Ammonia (total mg/L, pH 7.7	<30	<60	<60	<30
Ammonia (unionized, mg/L, pH 7.7)	<0.4	<0.8	<0.8	<0.4

[A] Environment Canada (1998 (207))

each species would be expected to encounter in the environment. *Eohaustorius estuarius* and *R. abronius*, the Pacific Coast amphipods, should be tested at 15°C. *Ampelisca abdita* should be tested at 20°C and *L. plumulosus* at 25°C.

A1.3.4.5 *Salinity*—The salinity of the water overlying the test sediment will vary depending on the selected test species. For routine testing, *A. abdita* and *R. abronius* should be tested at an overlying water salinity of 28 ‰, whereas *E. estuarius* should be tested at 20 ‰ . The target test salinity for *L. plumulosus* is 5 or 20 ‰ depending on the pore-water salinity. The recommended overlying salinity is 5 ‰ for test with sediment pore water from 1 to 10 ‰ or 20 ‰ for test sediments with pore water >10 ‰ . Alternatively, the salinity of the overlying water for toxicity tests with *E. estuarius* or *L. plumulosus* can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest). Depending on the objectives of the study, *E. estuarius* and *L. plumulosus* can be tested with overlying water salinity ranging from 1 to 32 ‰ . However, if tests are conducted with procedures different from those described in Table A1.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (sections 1.4 and 1.10). Pore-water salinity of each test sediment should be measured before the initiation of a test. Sediment pore water should be obtained by centrifugation. Alternatively, salinity can be measured before homogenization in the water that comes to the surface in the sample container as the sediment settles. The pore-water salinity of the test sediment should be within the salinity application range of the chosen amphipod species (Table A1.4). *Rhepoxynius abronius* cannot be tested when sediment pore-water salinities are <25 ‰ . Another species should be used for such sediments. *Ampelisca abdita*, *E. estuarius*, and *L. plumulosus* can be tested over the entire pore-water salinity range (that is, 1 to 34 ‰) when the recommended species-specific overlying salinity is used. PSEP (1995 (**191**)) outlines a procedure for adjusting the pore-water salinity of samples with a salinity <25 ‰ by adding appropriately saline overlying water to the test chamber on the day before the start of the test, mixing sediment and overlying water, and allowing the material to settle overnight under aeration. The resultant overlying water is either retained or about 75 % replaced with fresh dilution water at 28 ‰ (PSEP 1995 (**191**)). While this manipulation should result in an acceptable salinity for tests with *R. abronius*, the influence of this manipulation on the bioavailability of contaminants in the sediment sample is uncertain. See section 12.1.4.

A1.3.5 *Measurements and Observations:*

A1.3.5.1 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 time-weighted average of daily temperature readings must be within ± 2°C of the desired temperature. The instantaneous temperature must always be within ± 3°C of the desired temperature.

A1.3.5.2 Salinity, DO, and pH of the overlying water should be measured daily in at least one test chamber per treatment. Care should be taken not to disturb the sediment when sampling overlying water quality.

A1.3.5.3 Ammonia should be measured in overlying water towards the beginning (for example, Day 2) and towards the end of the test (for example, Day 8). Measurement of overlying water pH and temperature should accompany each ammonia measurement. Simultaneous measurements of ammonia, pH, and temperature in sediment pore water should be measured at the beginning of the test. Pore water should be extracted after the sediment has been press-sieved and homogenized. Samples of pore water should be obtained by centrifugation.

A1.3.5.4 Each test chamber should be examined at least daily during the 10-d test period to ensure that airflow to the overlying sea water is acceptable. The number of amphipods emerged from the sediment including those swimming in the water column and trapped in the air-water interface should be noted. Amphipods caught in the air-water interface should be gently pushed down into the water using a glass rod or pipette.

A1.3.6 *Ending a Test:*

A1.3.6.1 Laboratories should demonstrate the ability of their personnel to recover an average of at least 90 % of the organisms from control sediment. For example, test organisms could be added to control sediment and recovery could be determined after1 h (Tomasovic et al., 1994 (**208**)).

A1.3.6.2 The contents of the test chambers are sieved to isolate the test animals. The mesh size for sieving the contents of the test chambers should be no larger than 0.5 mm. Test water should be used for sieving. Material retained on the sieve should be washed into a sorting tray with clean test sea water. *Ampelisca abdita* are tube-builders and can be forced from their tubes for enumeration by slapping the sieve forceably against the surface of the water or by using a stream of water (for example, from a spray bottle or from a tube connected to a source of running water). *Eohaustorius estuarius*, *L. plumulosus*, and *R. abronius* are easily removed from the sediment by the sieving process. The sieve should be carefully

examined for any animals remaining, and in the case of *A. abdita*, also for any tubes (which may contain animals) remaining.

A1.3.6.3 Material that has been washed from the sieve into the sorting tray should be carefully examined for the presence of amphipods. A small portion of the material should be sorted through at a time, removing amphipods as they are found. Material from tests conducted with *A. abdita* will include tubes built by the amphipods during the test. The tubes should be carefully examined and teased apart under a dissecting microscope or magnifying glass because *A. abdita* will often remain in the tubes even after vigorous sieving. Numbers of live, missing, and dead amphipods should be determined and recorded for each test chamber. Missing animals are assumed to have died and decomposed during the test and disintegrated; they should be included in the number dead in calculations of the percent survival for each replicate treatment. Amphipods that are inactive but not obviously dead should be observed using a low-power dissecting microscope or a hand-held magnifying glass. Any animal that fails to exhibit movement (that is, neuromuscular twitch of pleopods or antennae) upon gentle prodding with a probe should be considered dead.

A1.3.7 *Test Data*—Survival is the primary endpoint recorded at the end of the 10-d sediment toxicity test with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius*. The ability of surviving amphipods to rebury in clean control sediment can be used to calculate effective mortality, that is, the sum of dead animals plus those survivors that fail to rebury. This endpoint has been used for *E. estuarius*, *L. plumulosus*, and *R. abronius*. If it is desired to determine reburial, surviving amphipods should be transferred to containers holding a 2-cm layer of 0.5-mm sieved control sediment and an overlying layer (22 cm) of test sea water. Salinity of the test sea water for reburial should be the same as that measured in the test chamber. The number of surviving amphipods unable to rebury in control sediment after1 h is recorded for each test chamber and is used to calculate effective mortality.

A1.4 Interpretation of Results

A1.4.1 Section 13.3 describes general information for interpretation of test results. The following sections describe species-specific information that is useful in helping to interpret the results of sediment toxicity tests with *A. abdita*, *E. estuarius*, *L. plumulosus*, and *R. abronius*.

A1.4.2 *Influence of Indigenous Organisms*—Indigenous organisms may be present in field-collected sediments. An abundance in the sediment sample of the test organism (McGee et al. 1999 (**41**)), or organisms taxonomically similar to the test organism, may make interpretation of treatment effects difficult. The presence of predatory organisms can also adversely affect test organism survival. For example, Redmond and Scott (1989 (**103**)) showed that the polychaete *Nephtys incisa* can consume *Ampelisca abdita* under toxicity test conditions.

A1.4.3 *Effect of Sediment Grain Size*—All four species show tolerance to most sediment types, with generally little effect on survival whether coarse-grained or fine-grained (that is, predominantly silt and clay) clean sediments are used. However, adverse effects due to the grain-size distribution of test sediment may occur when sediments that are either extremely sandy or fine depending on the species of amphipod used. In order to separate effects of sediment-associated contaminants from effects of particle size, an appropriate clean control and reference sediment should be incorporated into the test when test sediments are within the range of concern for each species. Alternatively, another species that is tolerant of the sediment extreme in question might be tested o conjunction with the chosen species. Ranges of concern are outlined below.

A1.4.3.1 *Ampelisca abdita*—Survival of *Ampelisca abdita* in sediment that is >95 % sand may elicit excess mortality, but this has not been quantified (USEPA 1994a (**1**), USEPA-USACE 2001(**2**)). Toxicity tests conducted with *A. abdita* on sediments that are >95 % sand should be conducted with a clean control sediment characteristic of that test sediment. (John Scott, personal communication USEPA, 1994a (**1**).)

A1.4.3.2 *Leptocheirus plumulosus*—In short-term exposures, juvenile *L. plumulosus* have been shown to be tolerant of a wide range of grain size. *Leptocheirus plumulosus* has exhibited >90 % survival in clean sediments ranging from about 100 % sand to about 100 % silt + clay (Schlekat et al., 1992 (**13**), USEPA 1994a (**1**)). See additional information provided in sections 12.1.3 and A2.4.3.

A1.4.3.3 *Eohaustorius estuarius—Eohaustorius estuarius* has exhibited acceptable (100 %) survival when exposed to clean sediments ranging from 0.6 to 100 % sand (USEPA 1994a (**1**)). Environment Canada (1998 (**181**)) reported that *E. estuarius* can tolerate up to 70 % clay in sediment toxicity tests. However, *E. estuarius* naturally inhabits sandy sediments, and a correlation between survival of *E. estuarius* and grain size has been reported by DeWitt et al. (1989 (**11**)) and by USEPA (1994a (**1**)) with increased mortality associated with increased proportions of fine-grained sediment. Therefore, it may be desirable to include clean control sediments with a range of particle sizes characteristic of those of the test sediment(s) in toxicity tests conducted with *E. estuarius*.

A1.4.3.4 *Rhepoxynius abronius—Rhepoxynius abronius* has been used to test sediments with a wide range of sediment grain sizes. However, *R. abronius* naturally inhabits clean, fine, sandy sediments, and a number of studies have shown some reduction in survival when this species is held in very fine-grained (predominantly silt and clay) sediment (DeWitt et al., 1988 (**192**); Long et al., 1990 (**29**); McLeay et al., 1991 (**193**); USEPA 1994a (**1**)). Therefore, when test sediments are predominantly silts or clays, the experimental design include a silt-clay control sediment with a range of particle sizes characteristic of the test sediment(s). Alternatively, when the particle size of test sediments are known, regression techniques can be used to evaluate potential effects of fines on *R. abronius* survival (DeWitt et al., 1988 (**192**)).

A1.4.4 *Effects of Pore-water Salinity*—The four amphipod species exhibit variability in their salinity tolerance ranges. There are two options available for laboratory sediment testing regarding the choice of overlying water salinity for a given sediment. The options are to either use the selected species-specific overlying water salinity for each test species (20 ‰ for *E. estuarius*, 5 or 20 ‰ for *L. plumulosus*, 28 to 32 ‰ for

A.abdita or *R. abronius*), or to choose another target salinity (for example, to match the salinity to that of the pore water). The range of pore-water salinities in which a given species can survive for 10 d when using species-specific overlying water salinities is the salinity application range. If tests are conducted with procedures different from those described in Table A1.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (sections 1.4 and 1.10).

A1.4.5 In either scenario, the potential for a toxic response due to salinity alone exists if a species is exposed to conditions outside of its range of tolerance. For estuarine sediments, it is very important to know the pore-water salinity of each sediment before testing is started, to choose a species that will not be affected by the pore-water salinity, and to use overlying water of an appropriate salinity.

A1.4.5.1 Salinity tolerance ranges for each species are as follows: *Ampelisca abdita*: 20 to 32 ‰ ;(USEPA 1994a (**1**)); *Eohaustorius estuarius*: 2 to 34 ‰ ; *Leptocheirus plumulosus*: 1.5 to 32 ‰ ; *Rhepoxynius abronius* : 25 to 32 ‰ . While there is some evidence of salinity-related stress for *E. estuarius* and *L. plumulosus* at salinity extremes, the breadth of salinity tolerance exhibited by these species (DeWitt et al., 1989 (**11**); Schlekat et al., 1992 (**13**); USEPA 1994a (**1**)) is most likely sufficient for application to the majority of sediments that may be encountered in an estuarine system. If it is desirable to have matching overlying and pore-water salinity from areas where pore-water salinities are 0 to 2 ‰, an organism that has been demonstrated to tolerate this salinity range should be used, either instead of or in addition. The amphipod *Hyalella azteca* is one such species (Test Method E1706). Likewise, sediments collected from areas of high salinity (that is, >32 ‰ for *L. plumulosus*) should probably utilize *A. abdita*, *E. estuarius*, or *R. abronius*.

A1.4.5.2 Salinity application ranges for each species are as follows: *Ampelisca abdita* with overlying water salinity of 28 to 32 ‰ : 0 to 34 ‰, (Weisberg et al., 1992 (**177**) ; USEPA 1994a (**1**)); *Eohaustorius estuarius* with overlying water salinity of 20 ‰ : <2 to 34 ‰ (DeWitt et al., 1989 (**11**); USEPA 1994a (**1**)); *Leptocheirus plumulosus* with overlying salinity of 20 ‰ : <1.5 to 32 ‰ (Schlekat et al., 1992 (**13**); USEPA 1994a (**1**) ; Emery et al. 1997 (**8**)) and *Rhepoxynius abronius* with overlying water salinity of 28 to 32 ‰ : 25 to 34 ‰ (Swartz et al., 1985 (**10**); Lamberson and Swartz, 1988 (**187**)).

A1.4.6 *Effects of Sediment-associated Ammonia*—Field-collected sediments may contain concentrations of ammonia that are toxic to amphipods. Water column no effect concentrations for the four amphipod species are presented in Table A1.4. If ammonia concentrations are above these concentrations, mortality occurring after 10 d may be due in part to effects of ammonia. Depending on test application, it may be desirable to lower the ammonia concentration by manipulating the test system before introduction of test organisms if measured ammonia in the overlying water is greater than the species specific no effect concentration (USEPA 1994a (**1**)). An errata sheet to USEPA (1994a (**1**)) suggests for dredged material testing under the Clean Water Act or the Marine Protection, Research, and Sanctuaries Act, the following procedure can be used. This procedure was described in a December 21, 1993 guidance memorandum issued by the U.S. EPA Office of Wetlands, Oceans and Watersheds, U.S. EPA Office of Science and Technology, and U.S. Army Corps of Engineers Operations, Construction, and Readiness Division. When ammonia is present at toxicologically important concentrations and when ammonia is not a contaminant of concern, the laboratory analyst can reduce ammonia in the sediment pore water to species-specific no-effect concentrations (Table A1.4). Ammonia levels in the pore water can be reduced by sufficiently aerating the sample and replacing two volumes of water per day. The analyst should measure pore-water ammonia periodically (for example, every 1 to 3 days) until it reaches the appropriate species-specific no-effect concentration. After placing the test organism in the sediment, the analyst should ensure that ammonia concentrations remain within an acceptable range by conducting the toxicity test with continuous flow or volume replacement of overlying water not to exceed two volumes per day. The purging of ammonia using this procedure may also remove other contaminants from the pore water of the sediment.

A2. PROCEDURE FOR CONDUCTING A *Leptocheirus plumulosus* 28-D TEST FOR MEASURING SUBLETHAL EFFECTS OF SEDIMENT-ASSOCIATED CONTAMINANTS

A2.1 Introduction

A2.1.1 *Leptocheirus plumulosus* has been used extensively to test the toxicity of estuarine or marine sediment. The choice of this amphipod species as a test organism is based on sensitivity to sediment-associated contaminants, availability and ease of collection and culturing, tolerance of environmental conditions (for example, temperature, salinity, grain size), ecological importance, ease of handling in the laboratory, and ease of measuring test endpoints. Additionally, this species is intimately associated with sediment by nature of its burrowing and feeding habits. *L. plumulosus* is tolerant of salinity values between >1 to 35 ‰ and sediment from fine- to coarse-grained. Field validation studies have shown that amphipods are absent or have reduced abundances at sites where toxicity has been demonstrated in laboratory tests. Amphipod sediment toxicity tests have been successfully performed for regulatory

and research purposes by numerous laboratories, including state and federal government agencies, private corporations, and academic institutions (see Section 1 for additional details).

A2.1.2 Guidance for conducting 10-d tests with *L. plumulosus* is described in Annex A1. Most standard whole sediment toxicity tests have been developed to produce a survival endpoint with potential for a sublethal endpoint (reburial) with some species. Methods that measure sublethal effects have either not been previously available or used routinely to evaluate sediment toxicity (Craig, 1984 (**28**); Dillon and Gibson, 1986 (**209**); Ingersoll and Nelson, 1990 (**210**); Ingersoll, 1991 (**211**) ; Burton et al., 1992 (**212**); USEPA 2000 (**206**); Test Method E1706). Most assessments of contaminated sediment rely on short-term lethality testing methods (for example, 10 d; USEPA-USACE, 1991 (**47**), 1998 (**48**)). Short-term lethality tests are useful in identifying "hot spots" of contamination, but may not be sensitive enough to evaluate moderately contaminated areas. However, sediment quality assessments using sublethal responses of benthic organisms, such as growth and reproduction, have been used to successfully evaluate moderately contaminated areas (Scott, 1989 (**12**); Niewolny et al. 1997 (**213**); DeWitt et al. 1997c (**214**)).

A2.1.3 The 28-d toxicity test with *L. plumulosus* is a test with a lethality endpoint and two sublethal endpoints: growth and reproduction. These sublethal endpoints have potential to provide a toxic response to chemicals that might not cause acute effects or significant mortality in a test. Sublethal response in 28-d exposures is also valuable for population modeling of contaminant effects. These data can be used for population-level risk assessments.

A2.1.4 A1.2 describes guidance for conducting the 28-d test with *L. plumulosus* that can be used to evaluate the effects of sediment contaminants on survival, growth, and reproduction. Refinement of these methods may be described in future editions of this standard, after additional laboratories have successfully used this method (section 15.6). These methods are based on procedures described in DeWitt et al. (1997a (**7**) ; 1997b (**26**) and Emery et al. (1997 (**8**)).

A2.1.5 Results of tests using procedures different from the procedures described in A2.2 may not be comparable, and these different procedures may alter contaminant bioavailability. Comparisons of results obtained using modified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sediment tests with estuarine or marine organisms. If tests are conducted using procedures different from those described in this standard, additional tests are required to determine comparability of results (sections 1.4 and 1.10).

A2.2 Procedure for Conducting a *Leptocheirus plumulosus* 28-d Test for Measuring Sublethal Effects of Sediment-associated Contaminants

A2.2.1 Recommended conditions for conducting a 28-d chronic sediment toxicity test with *L. plumulosus* are summarized in Table A2.1. A general activity schedule is outlined in Table A2.2. Decisions concerning the various aspects of experimental design, such as the number of treatments and water quality characteristics, should be based on the purpose of the test and the methods of data analysis (Section 13).

A2.2.2 The 28-d chronic sediment toxicity test with *L. plumulosus* is conducted at 25°C and a salinity of either 5 ‰ or 20 ‰ with a 16 h light : 8 h dark photoperiod at an illuminance of about 500 to 1000 lux (Table A2.1). Alternatively, the salinity of the overlying water can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest). If tests are conducted with procedures different from those described in Table A2.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (section 1.10).

A2.2.3 Test chambers are 1-L glass chambers containing 175 mL of sediment and about 775 mL of overlying seawater. Twenty neonate amphipods are added to each test chamber at the start of a test. Five replicate test containers per treatment are recommended for routine testing. Exposure is static-renewal with water exchanges and feeding three times per week, on Monday, Wednesday, and Friday. The test organisms are fed after water renewals. Overlying water can be culture, surface, site, or reconstituted water adjusted to the test salinity. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected. Requirements for test acceptability are summarized in Table A2.3.

A2.3 General Procedures

A2.3.1 *Sediment into Test Chambers:*

A2.3.1.1 The day before the addition of amphipods (Day-1), each test sediment, including control and reference sediment, should be homogenized among replicate beakers. This can be achieved by mixing, by stirring manually, or by using a rolling mill, feed mixer, or other apparatus (section 10.3) or by serially spooning out small aliquots of sediment to each test chamber. If a quantitative confirmation of homogeneity is required, replicate subsamples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size. The concentration of ammonia in pore water should also be measured at the start of the test.

A2.3.1.2 A 175-mL aliquot of sediment is added to each test chamber with five replicates per sediment treatment. It is important that an identical volume be added to each replicate test chamber; the volume added should provide a sediment depth of 2 cm in the test chamber. The sediment added to the test chamber should be settled by tapping the bottom or side of the test chamber against the palm of the hand or another soft object. Alternatively, sediment can be smoothed with a nylon, fluorocarbon, glass, or polyethylene spatula. Sediment known or suspected to be contaminated should be added to test chambers in a certified laboratory fume hood.

A2.3.2 *Addition of Overlying Water*—As test water is added, disruption of the sediment surface should be minimized. One way to accomplish this is by use of a turbulence reducer. Possible designs of turbulence reducer include a disk cut from polyethylene, nylon, or Teflon® sheeting (4 to 6 mil), or a glass petri dish attached (open face up) to a glass pipette. The

TABLE A2.1 Test Conditions for Conducting a 28-d Sediment Toxicity Test with *Leptocheirus plumulosus* (USEPA-USACE 2001 (2))

Parameter	Conditions
1. Test type:	Whole sediment toxicity test, static-renewal
2. Test sediment grain size:	>5 % silt and clay to<85 % clay
3. Test sediment pore-water salinity:	1 to 35‰
4. Overlying water salinity:	5 ‰ if pore water is 1 to 10 ‰, 20 ‰ if pore water is >10 to 35 ‰ ; Alternatively, the salinity of the overlying water can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest). If tests are conducted at a different salinity, additional tests are required to determine comparability of results (sections 1.4 and 1.10).
5. Test sediment pore-water ammonia:	< 60 mg/L (total mg/L, pH 7.7); < 0.8 mg/L (unionized mg/L, pH 7.7)
6. Test sediment pore-water sulfides:	Not established.
7. Temperature:	Daily limits: 25°C (±3°C); 28-d mean: 25°C (±2°C)
8. Light quality:	Wide-spectrum fluorescent lights
9. Illuminance:	500 to 1000 lux
10. Photoperiod:	16 h light: 8 h dark
11. Test chamber:	1-L glass beaker or jar with 10-cm inner diameter
12. Sediment volume:	175 mL (about 2-cm depth)
13. Sediment preparation:	Press-sieved through 0.25-mm sieve.
14. Overlying water volume:	Fill to 950 mL mark in test chamber (about 775 mL of water)
15. Renewal of overlying water:	3 times per week: siphon off and replace 400 mL
16. Source:	Laboratory cultures
17. Life stage and size:	Neonates: age-selected (<48 h old) or size-selected: retained between 0.25-mm and 0.6-mm mesh screens.
18. Number test organisms/chamber:	20
19. Number of replicate chambers/	5 for toxicity test; >2 additional replicate chambers for pore-water treatment: ammonia (Day 0 and Day 28)
20. Diet:	Days 0 to 13, 20 mg Tetramarine[4] per test chamber; Days 14 to 28, 40 mg Tetramarine[4] per test chamber.
21. Feeding schedule:	3 times per week (M-W-F) after water renewal.
22. Aeration and dissolved oxygen:	Aerate constantly with trickle flow of bubbles
Daily limits:	>3.6 mg/L (50 % saturation) 28-d mean: >4.4 mg/L (60 % saturation)
23. Overlying water:	Clean seawater, natural or reconstituted water; same source as used for culturing.
24. Overlying water quality and monitoring	Daily temperature in water bath or in an additional replicate chamber, daily frequency: minimum/maximum recommended; salinity, temperature, DO, and pH at test initiation and termination, and in one replicate per sediment treatment preceding water renewal during the test (three times per week); aeration rate daily in all containers; total ammonia on Days 0 and 28 in one replicate per treatment.
25. pH:	7.0 to 9.0 pH units
26. Pore-water quality:	Total ammonia, salinity, temperature, and pH of pore water from surrogate containers on Days 0 and 28; recommended in whole sediment before testing.
27. Test duration:	28 d
28. Test organism observations:	Observe condition and activity in each test chamber preceding water renewal (3 times per week).
29. Endpoints:	Survival, growth rate, and reproduction.
30. Test acceptability:	Minimum mean control survival of 80 %, growth and reproduction measurable in all control replicates, and satisfaction of performance-based criteria outlined in Table A2.3.

turbulence reducer is positioned just above the sediment surface and raised as water is added. It is convenient to mark each test chamber on the side at 950 mL and to fill with water to reach the mark. A turbulence reducer can be rinsed with clean water between replicates of a treatment, but a separate turbulence reducer should be used for each treatment. The test chambers should be covered, and placed in a temperature controlled water bath (or acceptable equivalent) in randomly assigned positions. Aeration is started when suspended sediment has settled (often overnight). A test begins when the test organisms are added to the test chambers (Day 0).

A2.3.3 *Initial Measurements*—On Day 0, water quality should be measured in all test chambers before adding amphipods to test chambers. If any water quality parameter is outside acceptable limits (Table A2.1), the problem should be corrected in all replicate containers of that treatment. The water quality characteristics should be re-measure and the test should be started once the water quality characteristics are acceptable. Aberrant pH values might be caused by characteristics of certain sediments and therefore may be impractical to correct.

A2.3.4 *Acclimation:*

A2.3.4.1 Test organisms should be cultured at a temperature near 25°C. Amphipod cultures held below 23°C need to be acclimated to test temperature of 25°C (± 3°C) before test initiation. Ideally, test organisms should be cultured in the same water that will be used in testing.

A2.3.4.2 Occasionally there is a need to perform evaluations at a temperature or salinity different than those recommended in Table A2.1. Under these circumstances, it may be necessary to acclimate organisms to the desired test temperature or salinity to prevent thermal shock that could result when organisms are moved immediately from the culture temperature or salinity to the test temperature or salinity. Reproduction and growth rates in cultures may be greatly reduced at temperatures <20°C. However, reproduction and growth is not effected at a salinity ranging from 5 ‰ and 20 ‰ (DeWitt et al., 1997a (7)). Acclimation can be achieved by exposing organisms to a gradual change in temperature or salinity. However, the rate of change should be relatively slow to prevent shock. A change in temperature or salinity not exceeding 3°C or 3 ‰ per 24-h period is strongly recommended (Section 12). Tests at temperatures other than 25°C need to be preceded by studies to determine expected performance under alternate conditions.

A2.3.5 *Addition of Amphipods:*

A2.3.5.1 The test is initiated when amphipods are added to the test chambers. See section 12.5 for procedures for obtaining neonates for testing. Amphipods should be impartially selected

TABLE A2.2 General Activity Schedule for Conducting a 28-d Sediment Toxicity Test with *Leptocheirus plumulosus* (USEPA-USACE 2001 (2))

Day	Activity
Preparation	
Pretest	Start or renew cultures about 6 to 8 weeks in advance of test initiation. Increase culture water temperature to about 25°C about 2 weeks in advance of test initiation.
Pretest	Determining pore-water salinity of test sediment and acclimate *L. plumulosus* cultures to overlying water salinity to be used in testing.
Day-1	Layer sediment in test chambers, add overlying water. Measure pore-water total ammonia in whole sediment and begin purging procedures, if appropriate (section A2.4.5). Measure tare weight of weigh boats for dry weights. Set up positive control reference-toxicity test chambers if appropriate.
Initiation	
Day 0	Measure pore-water total ammonia, temperature, salinity, and pH in an additional replicate chamber. Measure salinity, temperature, DO, and pH in all test chambers. If water quality parameters are within test ranges, proceed with initiation; if not, correct problem and re-measure water quality. Obtain neonate test organisms, initiate test, and initiate positive control reference toxicant test if conducted. Only feed if a Monday, Wednesday, or Friday. Prepare 3 sets of 20 neonates for initial weight of growth rate endpoint; rinse in deionized water; dry overnight at 70°C, and weigh or measure length on Day 1 or later.
Positive Control Reference-toxicity Test	
Day 1 to 3	Measure and record water quality parameters in one replicate test chamber from each positive control treatment.
Day 4	Measure water quality parameters and record observations of amphipod activity in all positive control test chambers. Terminate the positive control references-toxicity control test if conducted.
Maintenance of 28-d Test	
Daily	Check aeration in all test chambers and test temperature (water bath, environmental chamber, or in an additional replicate chamber). If aeration is interrupted in a test chamber, measure and record DO before resumption of aeration. Check photoperiod controllers.
3 Times per Week (M-W-F)	Measure water quality in one replicate test chamber per sediment treatment. Record observations of amphipod activity and condition of sediment and water in all test chambers. Siphon off and replace 400 mL of water in all test chambers. Add food to all test chambers.
Termination of 28-d Test	
Day 28	Measure salinity, temperature, DO, and pH in all test chambers. Measure tare weight of weight boats for dry weight measurements. Terminate 28-d test: sieve adults and offspring from sediment, count surviving adults, prepare adults for drying, and dry to constant weight at 70°C. Count offspring, or preserve and stain offspring.
Day 29 or later	Measure dry weight or length of adults. If offspring were preserved, count them.

and placed in transfer containers (small dishes or eye cups) containing a small amount of test water. The number of amphipods in each dish should be verified by recounting before organisms are added to test chambers. To facilitate recounting, amphipods may be distributed to test chambers in batches of 5 or 10 instead of the full complement of 20. Because neonates are very small, caution should be taken to ensure that each test chamber receives all 20 amphipods at test initiation. The distribution of amphipods to the test chambers needs to be done in an impartial fashion. Animals need to be added to test chambers as soon as possible following their collection to minimize handling stress and exposure to temperature changes. Three impartially selected sets of 20 neonates for initial weight determination should be isolated at the start of the test.

A2.3.5.2 To facilitate the initiation process, aeration should be stopped in test chambers immediately before adding the neonates. Sediment in test chambers should not be disrupted during the initiation procedure. Neonates from a transfer container should be poured into a test chamber. Any neonates remaining in transfer containers can be washed immediately into the test chamber using a gentle stream of water at appropriate temperature and salinity. Neonates trapped at the surface water can be submerged by using a blunt probe or by gently dribbling a few drops of test or culture water onto the amphipod from above. A disk of 6-mil polyethylene, nylon, or Teflon® can be used on the water surface to minimize disruption of the sediment surface, if necessary. Rinse the disk after amphipods are added to ensure that none of the amphipods have stuck to the disk. The disk should be removed once the amphipods have been introduced. A separate disk should be used for each treatment to avoid cross contamination. Aeration may be discontinued for up to 1 to 2 h to allow the amphipods to burrow into the sediment. Aeration should then continue for the remainder of the test.

A2.3.5.3 After the test organisms have been added, the test chambers should be examined for individuals that did not burrow into the sediment and might have been stressed or injured during the isolation, counting, or initiation processes. Injured or stressed test organisms will not burrow into sediment and should be removed. Neonates that have not burrowed within 1 h should be replaced with test organisms from the same sieved population, unless they are repeatedly burrowing

TABLE A2.3 Test Acceptability Requirements for a 28-d Sediment Toxicity Test with *Leptocheirus plumulosus* (USEPA-USACE 2001 (2))

A. It is recommended for conducting the 28-d test with *L. plumulosus* that the following performance criteria are met:
1. Neonate *L. plumulosus*, size-selected (retained between 0.25-mm and 0.6-mm screens) or age selected (<24-h old), are used to initiate the test(s).
2. Average survival of amphipods introduced at the start of the test in the negative control sediment must be greater than or equal to 80 % at the end of the test, with no single replicate having 60 % survival or less.
3. Measurable growth and reproduction should be observed in all replicates of the negative control treatment.

B. Performance-based criteria for culturing *L. plumulosus* include the following:
1. Laboratories should perform periodic 96-h water-only reference-toxicity tests (at a minimum, one test every six months) to assess the sensitivity of culture organisms (section 11.16).
2. Records should be kept on the frequency of restarting cultures.
3. Laboratories should record the pH and ammonia of the culture water at least quarterly. Dissolved oxygen and salinity should be measured weekly. Temperature should be recorded daily.
4. Laboratories should characterize and monitor background contamination and nutrient quality of food if problems are observed in culturing or testing organisms.

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 solvent control used must not adversely affect test organisms.
5. Natural physico-chemical characteristics of test sediment collected from the field should be within the tolerance limits of the test organisms.
6. Salinity, pH, and DO, in the overlying water, ammonia in pore water and test sediment grain size should be within test condition limit of the test species (Table A2.1), or else effects of the variables need to be considered during interpretation of test results.
7. The time-weighted average of daily temperature readings must be within +2°C of the desired temperature. The instantaneous temperature must always be within +3°C of desired temperature.
8. The time-weighted average of daily salinity readings should be within ± 2 ‰ of the selected salinity and the instantaneous salinity readings should be ± 3 ‰ of the selected salinity.

into the sediment and immediately emerging in an apparent avoidance response. In that case, the amphipods are not replaced. The number of amphipods that are replaced in each test chamber should be recorded.

A2.3.6 *Test Conditions:*

A2.3.6.1 Test limits for the 28-d *L. plumulosus* test are provided in Table A2.1. Test sediments with characteristics that exceed these limits are subject to noncontaminant effects that should to be considered during interpretation of test results.

A2.3.6.2 *Aeration*—The overlying water in each test chamber should be aerated continuously after an initial settling period, except during introduction of the test organisms. Filtered, dry, clean air should be bubbled through a glass or plastic pipette via plastic tubing (about 3 bubbles/sec). The tip of the pipette should be suspended 2 cm to 3 cm above the surface of the sediment so that it does not disturb the sediment surface. The concentration of DO in the water overlying the sediment in the test chambers is maintained at or near saturation by gentle aeration. Ideally, air is bubbled through the water at a rate that maintains a high percentage of saturation (for example, about 90 %) but does not disturb the sediment surface. If air flow to one or more test chambers is interrupted (that is, for more than 1 h), DO should be measured in those test chambers to determine whether DO concentrations have fallen below 4.4 mg/L. The 28-d mean should be >4.4 mg/L DO, and daily DO measurements should be >3.6 mg/L (50 % saturation). Results may be unacceptable for test chambers in which aeration is interrupted or DO concentrations fall to below 50 % of saturation.

A2.3.6.3 *Lighting*—Laboratory lighting should be maintained on a 16 h light: 8 h dark photoperiod cycle throughout the test at an intensity of 500 to 1000 lux.

A2.3.6.4 *Feeding and Water Renewal*—A Tetramarine[4]-only diet is recommended for the 28-d sediment toxicity test with *L. plumulosus*. With this diet, 400 mL of overlying water is replaced three times per week (Monday-Wednesday-Friday), after which a Tetramarine[4] slurry is delivered to each chamber in 1-mL aliquots. Water removal and replacement should be completed using procedures that minimize disturbance to sediment in the test chambers. Water can be removed by siphoning through a tube with fine-meshed screening over the intake to prevent uptake of amphipods. A pump can also be used to remove water. Water should not be poured from test chambers because this practice can resuspend and disturb the sediment. A separate turbulence-reducer should be used for each treatment when water is replaced to avoid cross contamination (A2.3.2). Tetramarine[4] is fed at a rate of 20 mg per test chamber between Days 0 to 13 and 40 mg per test chamber between Days 14 to 28. To prepare the slurry, Tetramarine[4] is finely ground with a food mill (blender, mortar and pestle, or a similar device) and sieved through a 0.25-mm screen. Test water is added to the appropriate amount of Tetramarine[4], and the slurry is mixed on a stir plate for 15 min. USEPA-USACE (2001(**2**)) provides a sample calculation for preparation of food rations. The slurry is prepared fresh for each use and needs to be mixed continuously during feeding to prevent the Tetramarine[4] from settling.

A2.3.6.5 Laboratory experimentation has shown that food ration can affect the response of test animals to sediment-associated contaminants. The food ration of Tetramarine[4] recommended in this standard was evaluated with two other food rations in an experiment in which test animals were exposed to sediments spiked with PCB29 at concentrations between 15 and 240 mg/L (USEPA-USACE 2001 (**2**)). Three separate feeding rates were evaluated at each PCB29 concentration included 30 mg/60 mg (Days 0 to 13/Days 14 to 18), 20 mg/40 mg and 10 mg/20 mg per test chamber. Significant reductions in survival and growth were evident only in the highest PCB29 concentration for each of the food rations. Decreased reproduction was also evident at 240 mg/L PCB29 at each food ration as well as at 120 mg/L for the 20 mg/40 mg and 10 mg/20 mg rations (USEPA-USACE 2001 (**2**)). Given the generally lower reproductive rates observed at the lowest food ration, the 20 mg/40 mg feeding rate is recommended for use in this standard.

A2.3.6.6 *Water Temperature*—The test temperature was selected to approximate summertime temperature experienced by *L. plumulosus* in the wild (Holland et al., 1988 (**182**); McGee, 1998 (**183**)). The test temperature is 25°C with a daily maximum range of ± 3°C and a 28-d weighted mean of 25°C ± 2°C. Water used for renewal of test chambers should be adjusted to test temperature before use in renewals.

A2.3.6.7 *Salinity*—The target test salinity for *L. plumulosus* is 5 or 20 ‰ depending on the pore-water salinity. The recommended overlying salinity is 5 ‰ for test with sediment pore water from 1 to 10 ‰ or 20 ‰ for test sediments with pore water > 10 ‰ . The 28-d mean salinity values should deviate no more than 2 ‰ from the recommended salinity (for example, 5 ‰ or 20 ‰). Pore-water salinity of each test sediment should be measured before the initiation of a test. Sediment pore water can be measured in water overlying sediment in sample containers before homogenization of sediment. Pore-water salinity can also be obtained by centrifugation (Section 10). Alternatively, the salinity of the overlying water can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest). If tests are conducted with procedures different from those described in Table A2.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (sections 1.4 and 1.10).

A2.3.7 *Measurements and Observations:*

A2.3.7.1 Temperature should be measured at least daily in an additional replicate chamber or from the water bath or environmental chamber. The temperature of the water bath or a test chamber should be continuously monitored with minimum and maximum temperature recorded daily. An additional replicate container identical to test containers is recommended for continuous temperature monitoring. The time-weighted average of daily temperature readings must be 25°C ± 2°C. The instantaneous temperature must always be within ± 3°C of the desired temperature.

A2.3.7.2 Salinity, DO, temperature, and pH of the overlying water should be measured three times per week in at least one test chamber per treatment before renewal of water. Care should be taken not to disturb the sediment when sampling overlying water quality.

A2.3.7.3 Total ammonia should be measured in overlying and pore water at test initiation (Day 0 or Day -1 for pore water) and at test termination (Day 28). Salinity, pH, and temperature should be measured with each ammonia measurement. Simultaneous measurements of ammonia, salinity, pH, and temperature in sediment pore water should be taken before test initiation. If test sediments are sieved, pore-water samples for ammonia should be collected before and after sieving. Pore water can be obtained by centrifugation or from overlying water in sample containers (before pretest homogenization). If ammonia levels exceed recommended limits (Table A2.1), then ammonia reduction procedures are advisable before test initiation. However, if ammonia is the chemical of concern in the test sediments, pore-water ammonia concentrations should not be deliberately manipulated.

A2.3.7.4 Each test chamber should be examined daily to ensure that airflow to the overlying water is acceptable. Daily checks for amphipods trapped at the water surface are recommended for the first three days of a test. Amphipods caught in the air-water interface should be gently pushed down into the water using a blunt glass probe or drops of dilution water. The number of amphipods swimming in the water column and trapped in the air-water interface should be noted and amphipods submerged before each water renewal. The number of apparently dead test organisms should be noted, but organisms should not be removed or otherwise disturbed during the test. Exuviae may be mistaken for dead amphipods; therefore, care should be taken in identifying animals as dead.

A2.3.8 *Ending a Test:*

A2.3.8.1 The contents of each test chamber are sieved to isolate the test organisms. The mesh sizes for sieving the contents of the test chambers is 0.5 to 0.6 mm to isolate adults and 0.25 mm to isolate offspring. The 0.6-mm sieve should not be stacked atop the 0.25-mm sieve for this process. Test water should be used for sieving. Material retained on each sieve should be washed into a sorting tray with clean test water. *L. plumulosus* are easily removed from the sediment by the sieving process.

A2.3.8.2 Material that has been washed from the sieve into a sorting tray should be carefully examined for the presence of amphipods. A small portion of the material should be sorted through at a time, and amphipods should be removed as they are found. Amphipods and residual sediment retained on the 0.25-mm sieve should be rinsed briefly with freshwater to remove salts and washed into a labeled sample jar using 70 % alcohol (either ethyl or isopropyl). Use of a wide funnel supported by a ring stand facilitates this process. Because offspring are very small, care is needed to transfer all organisms from the screen to the sample jar. Add sufficient 70 % alcohol to preserve the amphipods, and add about 3 mL of rose bengal solution (about 1 g/L) to stain the organisms. Offspring can be counted on test termination day, but waiting 2 to 3 d allows the amphipods to be more darkly stained.

A2.3.8.3 *Survival*—Numbers of live and dead adult amphipods should be determined and recorded for each test chamber. Missing adult organisms are assumed to have died, decomposed, and disintegrated during the test; they should be included in the number dead in calculations of the percentage survival for each replicate treatment. Amphipods that are inactive but not obviously dead are observed using a low-power dissecting microscope or a hand-held magnifying glass. Any organism that fails to exhibit movement (that is, neuromuscular twitch of pleopods or antennae) upon gentle prodding with a probe should be considered dead. An independent count of the number of isolated amphipods that are dead, alive, or moribund should be made in 10 % of replicates by a second observer. Based one the experience of one laboratory, the intralaboratory median CV for survival (sample size of 88 treatments) can be expected to be 11 % (DeWitt et al. 1997b (**26**); section 15.6.1). Based on one study involving 10 laboratories, the interlaboratory CV for survival ranged from 4 to 19 % (DeWitt el a. 1997b (**26**); section 15.6.2). It should be expected that intralaboratory CV for survival will decrease

over time as a laboratory gains experience using this method. Similarly, the interlaboratory CV for survival should decrease from reported values here as more laboratories gain experience using this method.

A2.3.8.4 *Growth Rate*—Growth rate of amphipods can be reported as daily change of average individual length or weight. However, measuring length is more laborious and therefore more expensive than measuring weight to determine growth rate, and does not result in an increase in sensitivity in *L. plumulosus* 28-d test (DeWitt et al., 1997a (**7**)). Dry weight of amphipods can be determined as follows: *(1)* transferring the archived amphipods from a replicate out of the preservative into a crystallizing dish; *(2)* rinsing amphipods with deionized water; *(3)* transferring these rinsed amphipods to a preweighed aluminum pan; *(4)* drying these samples to constant weight at 60°C; and *(5)* weighing the pan and dried amphipods on a balance to the nearest 0.01 mg. Average dry weight of individual amphipods in each replicate is calculated from these data. Due to the small size of the amphipods, caution should be taken during weighing 20 dried amphipods after 28-d sediment exposure may weigh less than 25 mg). The average per-capita dry weight of adult amphipods for each replicate is the difference between the tarred weight of the boat and the total weight of the boat plus dried amphipods, divided by the number of amphipods in the weigh boat. The growth rate endpoint (mg/d) is the difference between per capita adult and neonate dry weights, divided by 28 d. In other words, for each replicate, calculate: Growth Rate (mg/individual/day) = (mean adult dry weight - mean neonate dry weight)/28 (note that this pooled weight of neonates is typically very small). Weigh pans need to be carefully handled using powder-less gloves and the balance should be calibrated with standard weights with each use. Forceps can also be used to handle the weigh pans. Use of small aluminum pans will help reduce variability in measurements of dry weight. Weigh boats can also be constructed from sheets of aluminum foil. Amphipod body length (±0.1 mm) can be measured from the base of the first antennae to the tip of the third uropod along the curve of the dorsal surface. The use of a digitizing system and microscope to measure length has been described in Kemble et al. (1994 (**50**)) for *Hyalella azteca* and DeWitt et al. (1992a (**6**) and 1997a (**7**)) for *Leptocheirus plumulosus*. Based on the experience of one laboratory, the intralaboratory median CV for growth (sample size of 87 treatments) can be expected to be 3 % (DeWitt et al. 1997b (**26**); section 15.6.1). Based on one study involving 10 laboratories, the interlaboratory CV for growth ranged from 14 to 38 % (DeWitt el a. 1997b (**26**); section 15.6.2). It should be expected that intralaboratory CV for growth rate will decrease over time as a laboratory gains experience using this method. Similarly, the interlaboratory CV for growth rate should decrease from reported values here as more laboratories gain experience using this method.

A2.3.8.5 *Reproduction*—The offspring should be counted within 2 weeks of terminating the test. It may be possible to count the offspring the day the experiment is broken down. If not, preserve offspring in 70 % alcohol (either ethyl or isopropyl). Transfer preserved, stained offspring to a fine screen (<0.25-mm mesh) and rinse with freshwater to remove alcohol and excess stain. Rinse the live or preserved neonates into a shallow dish and count them under magnification, such as a dissecting microscope. Record the number of offspring. For QA, 10 % of the samples should be recounted by a second analyst. The reproduction endpoint is calculated as the number of offspring per living adult. Based on the experience of one laboratory, the intralaboratory median CV for reproduction (sample size of 88 treatments) can be expected to be 18 % (DeWitt et al. 1997b (**26**; section 15.6.1). Based on one study involving 10 laboratories, the interlaboratory CV for survival ranged from 35 to 102 % (DeWitt el a. 1997b (**26**; section 15.6.2). It should be expected that intralaboratory CV for reproduction will decrease over time as a laboratory gains experience using this method. Similarly, the interlaboratory CV for reproduction should decrease from reported values here as more laboratories gain experience using this method.

A2.3.9 *Control Performance Issues and Revisions to the Method*—The *Leptocheirus plumulosus* 28-d sediment toxicity test, like all experimental systems, is subject to occasional failures. Because the *L. plumulosus* 28-d sediment toxicity test is more complex and of longer duration than the 10-d sediment toxicity tests described in Annex A1, there are more opportunities for problems to occur in this long-term test than in the short-term tests. Problems with the test are most readily detected by failure to meet test acceptability criteria in the control treatment (Table A2.1 and Table A2.3), such as mortality <20 % or failure of amphipods to grow or reproduce. Test failures usually can be attributed to a failure to maintain one or more test requirements described in Table A2.1 and Table A2.3; however, tests sometimes fail inexplicably. Possible causes for unaccountable test failures have included overfeeding (for example, leading to anoxia or increased production of hydrogen sulfide), poor health of test animals (that is, culture failure), or accidental introduction of toxic materials into test chambers. Scientists from the USEPA and the USACE observe that the frequency of failure decreases as the laboratory and staff using the test gain more experience through conducting the test; however, neither agency has explicit data on the frequency of failure. Users of this test should be aware of this possibility and prepare for the possibility to rerun the test on occasion. The method for the *L. plumulosus* 28-d sediment toxicity test will be revised as new experimental data reveal test conditions that reduce the probability of possible test failure.

A2.4 Interpretation of Results

A2.4.1 This section describes information that is useful in helping to interpret the results of sediment toxicity tests with *L. plumulosus*. Section 13 provides additional information on analyses and reporting of toxicity test data.

A2.4.2 *Influence of Indigenous Organisms*—Indigenous organisms may be present in field-collected sediment. The presence of organisms taxonomically similar to the test organism or the presence of the test organisms in the sample (McGee et al. 1999 (**41**)) can make interpretation of treatment effects difficult. Predatory organisms can adversely affect test organism survival. For example, Redmond and Scott (1989 (**103**)) showed that the polychaete *Nephtys incisa* can consume

amphipods under test conditions. All control, reference, and test sediment should be press-sieved through 0.25-mm mesh to avoid these complications. If test sediment is not sieved, the number and species of indigenous organisms should be determined to better interpret results.

A2.4.3 *Effects of Sediment Grain Size—L. plumulosus* tolerates a wide range of sediment types. There is generally little effect on survival, growth rate, or reproduction when coarse-grained (sand) or fine-grained (predominantly silt and clay) sediment is used. See section 12.1.3 for additional detail. In some studies, *L. plumulosus* has exhibited >90 % survival in clean sediment ranging from nearly 100 % sand to nearly 100 % silt + clay (Schlekat et al., 1992 (**13**), USEPA 1994a (**1**)). However, adverse effects can occur in sediment with very high levels of clay or sand. Laboratory studies have shown significant reduction in survival when clay content exceeded 84 %, and survival, growth and reproduction were significantly reduced in 100 % sand (Emery et al., 1997 (**8**)). Results have been equivocal from controlled tests with mixed grained sediments (between 10 % and 90 % silt/clay). Emery et al. (1997 (**8**)) found an increase in growth as sediment coarseness increased up to 75 % sand. DeWitt et al. (1997a (**7**)) reported enhanced growth in finer-grained sediment as compared with more coarse-grained material, but the difference in growth was not considered to be biologically significant (DeWitt et al., 1997a (**7**)). Therefore, *L. plumulosus* should be tested with sediment with silt/clay content between 5 % and 85 % (Table A2.1). If sediment characteristics exceed these bounds, an appropriate clean control and reference sediment should be incorporated into the test to separate effects of sediment-associated contaminants from effects of particle size.

A2.4.4 *Effects of Pore-water Salinity:*

A2.4.4.1 The range of salinity in which a given species can survive when the overlying water salinity is matched to that of the pore-water salinity is the salinity tolerance range. The potential for a toxic response caused by salinity alone exists if a species is exposed to conditions outside of its range of tolerance. For estuarine sediment, it is important to know the pore-water salinity of each sediment before testing is started and to use overlying water of an appropriate salinity. *L. plumulosus* is not recommended for testing with freshwater sediments (<1 ‰ pore-water salinity or with sediments having pore-water salinity >35 ‰ until further testing is completed to confirm acceptable response in organisms (DeWitt et al., 1997a (**7**)). This standard recommends use of standard salinity of overlying water for testing (that is, 5 ‰ or 20 ‰ ; Table A2.1).

A2.4.4.2 *L. plumulosus*, a euryhaline species, can survive and thrive in a wide range of salinity conditions. The salinity tolerance and application range for this amphipod is 1 to 35 ‰ (DeWitt et al., 1989 (**11**); DeWitt et al., 1992a (**6**); Schlekat et al., 1992 (**13**); DeWitt et al., 1997a (**7**)). Although there is some evidence of salinity-related stress for *L. plumulosus* at salinity extremes, the breadth of salinity tolerance exhibited by this species is most likely sufficient for application to the majority of sediments that might be encountered in an estuarine system (that is, pore-water salinity from 1 to >30 ‰).

A2.4.4.3 This method recommends testing with an overlying water salinity of either 5 or 20 ‰ ; the choice of overlying water salinity is dependant on the pore-water salinity of test sediment. Alternatively, the salinity of the overlying water can be adjusted to a selected target salinity (for example, one representative of the salinity regime at the site of interest). If tests are conducted with procedures different from those described in Table A2.1 (for example, different salinity, lighting, temperature, feeding conditions), additional tests are required to determine comparability of results (sections 1.4 and 1.10).

A2.4.4.4 Although matching overlying and pore-water salinity values in test containers might be appropriate for some study designs, this practice is logistically complicated and normally impractical to accomplish. Acclimation of amphipod cultures to the appropriate salinity is required. Moreover, if sediment samples to be tested have different pore-water salinity values, care needs to be exercised to ensure that renewals are completed with water of the appropriate salinity.

A2.4.5 *Effects of Sediment-associated Ammonia:*

A2.4.5.1 Field-collected sediment may contain concentrations of pore-water ammonia that are toxic to amphipods. The water-only NOEC for *L. plumulosus* is 60 mg/L (USEPA, 1994a (**1**)). If ammonia concentrations are above this value at test initiation, mortality may be due in part to effects of ammonia. Depending on test application, it might be desirable to lower the ammonia concentration by manipulating the test system before introduction of test organisms if measured ammonia in the pore water or overlying water is greater than the NOEC. However, if ammonia is the chemical of concern in the test sediments, pore-water ammonia concentrations should not be deliberately manipulated. If sediment toxicity tests are conducted to evaluate the acceptability of dredged material for disposal, the manipulations could be performed. Section 13.3.6 references methods for conducting TIEs to determine whether ammonia is contributing to the toxicity of sediment samples. Manipulations involve flushing the test system by renewing a specified amount of overlying water until ammonia concentrations are reduced (section A1.4.5). The effects of dilution of ammonia on pore-water concentration is not known. Due to this uncertainty, one option could be to monitor pore-water concentrations.

A2.4.5.2 If ammonia is of concern to the regulatory application associated with the sediment toxicity test, overlying water should be sampled about 1 cm above the sediment surface before introduction of test organisms on Day 0. Pore-water ammonia should be measured when sediment samples are prepared for testing. If both the pore water and overlying water ammonia concentrations are <60 mg/L, then the test may proceed normally. If the ammonia concentration is >60 mg/L in a given sample, then ammonia level can be reduced by aerating the sample to saturation and replacing 2 volumes of overlying water per day. Purging pore-water ammonia (up to 60 mg/L) from test sediments before starting the toxicity test, and employing the routine replacement of overlying water in each test chamber every other day (M-W-F) did result in a consistently reduced pore-water ammonia concentration throughout the 28 days from about 60 mg/L to

about 1 mg/L (DeWitt et al., 1997a (7)). Similar results were obtained by other researchers (Moore et al. 1997 (27); Moore et al. 1995 (215)). The analyst should measure the pore-water ammonia concentration each day until it is <60 mg/L. The pore-water ammonia threshold for the chronic sediment toxicity test was based on 28-d exposures of the amphipods to sediments with experimentally-elevated pore-water ammonia (up to 60 mg/L), employing the specified purging technique before starting the toxicity test exposure, and employing the routine replacement of overlying water (M-W-F; DeWitt et al., 1997a (7)). No lethal or sublethal toxicity was observed in this experiment at any one of the tested pore-water ammonia concentrations, which is most likely caused by loss of ammonia from the test system due to diffusion of pore-water ammonia from the sediments to the overlying water and the replacement of the overlying water three times per week. Because additional replicate containers are required for pore-water measurements, a minimum of two additional replicate containers are required (one for Day 0 and one for Day 28). Additional replicate containers should be prepared if pore-water ammonia levels are high enough to require several successive days for pore-water ammonia reduction. When ammonia concentrations are reduced to <60 mg/L, testing should be initiated by adding test organisms.

A2.4.6 *Hydrogen Sulfide*—Hydrogen sulfide occurs naturally in anoxic marine sediments. Sims and Moore (1995 (216)) conducted an extensive review of the literature that focused on the effects of hydrogen sulfide on benthic organisms. Sims and Moore (1995 (216)) reported that tube-building amphipods circulate oxygenated water through their burrows, thus reducing or eliminating exposure to pore-water hydrogen sulfide. In acute experiments, however, dissolved sulfides have been shown to be toxic to marine amphipods *R. abronius* and *E. estuarius* (48-h LOECs of 1.47 and 1.92 mg/L total sulfide respectively; Knezovich et al. 1996 (217)). Currently, no data exist regarding the sensitivity of *L. plumulosus* to hydrogen sulfide in 28-d exposures. Additional information on the tolerance of aquatic organisms to sulfides can be found in Bagarinao (1992 (218)).

A2.4.7 *Total Organic Carbon (TOC)*—Test sediment TOC content can vary greatly, ranging from near 0 to >10 %. The amount of TOC can affect test organism survival, growth, and reproduction. Limited evidence suggests that the *L. plumulosus* chronic test is tolerant to most TOC concentrations; however, Scott et al. (1996 (219)) reported that growth and reproduction may be lower in uncontaminated field sediments having <2 % TOC concentrations. An analysis of organism response over a wide range of sediment TOC was completed by DeWitt et al. (1997b(26)) using reference sediment data from two studies. No effect on survival, growth, or reproduction was detected for sediments with TOC concentrations ranging from 1 to 7 % TOC. There was some evidence of significantly decreased survival, growth, and reproduction in <1 % TOC sediments. No data were available for test sediments with TOC >7 %. Therefore until additional data are generated, if test sediment TOC concentrations are <1 % or >7 %, a TOC control or reference sediment with similar TOC should be tested concurrently.

A2.4.8 *Future Research*—Research to find methods that reduce the variability of the growth rate and reproduction endpoints could lead to improvements in the statistical power of the *L. plumulosus* chronic toxicity test. A second "round-robin" study using the revised feeding regime and using only laboratories with considerable experience running this toxicity test, could provide improved estimates of the interlaboratory accuracy and precision of each endpoint. Additional research is needed to evaluate the relative toxicological sensitivity of the lethal and sublethal endpoints to a wide variety of chemicals spiked in sediment and to mixtures of chemicals in sediments from contaminant gradients in the field. Additional research is needed to evaluate the ability of the test's lethal and sublethal endpoints to estimate the responses of populations and communities of benthic invertebrates to contaminated sediments. Additional research is also needed to link the toxicity endpoints to a field validated population model of *L. plumulosus* (McGee and Spencer 2001 (220), Spencer and McGee 2001 (221)) which would provide additional ecological relevant interpretative guidance for the toxicity test.

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SUMMARY OF CHANGES

The primary technical changes from the previous version of this standard (E1367-99) are summarized in this section.

(*1*) The information on conducting 10-d sediment toxicity tests with estuarine and marine amphipods has been updated based on information presented in USEPA (1994a (**1**)).
(*2*) The information on conducting 28-d sediment toxicity tests with *Leptocheirus plumulosus* has been included based on information presented in USEPA-USACE (2001 (**2**)).
(*3*) The general format of the standard has been revised to be consistent with Test Method E1706.