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Standard Guide for Conducting Acute, Chronic, and Life-Cycle Aquatic Toxicity Tests with Polychaetous Annelids[1]

This standard is issued under the fixed designation E1562; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers procedures for obtaining data concerning the adverse effects of a test material added to marine and estuarine waters on certain species of polychaetes during short- or long-term continuous exposure. The polychaete species used in these tests are ~~taken~~ either field collected or from laboratory cultures and exposed to varying concentrations of a toxicant in static or static-renewal conditions. These procedures may be useful for conducting toxicity tests with other species of polychaetes, although modifications might be necessary.

1.2 Modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, the results of tests conducted using unusual procedures are not likely to be comparable to those of many other tests. Comparisons of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting acute, chronic, or life-cycle tests with other species of polychaetes.

1.3 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, and known or unknown mixtures. With appropriate modifications, these procedures can be used to conduct these tests on factors such as temperature, salinity, and dissolved oxygen. These procedures can also be used to assess the toxicity of potentially toxic discharges such as municipal wastes, sediments/soils, oil drilling fluids, produced water from oil well production, and other types of industrial wastes. An LC50 (medial lethal concentration) ~~is~~ may be calculated from the data generated in each acute and chronic toxicity ~~test. Reproductive success and the number of offspring produced~~ test when multiple concentrations are tested. Growth, determined by a change in measured weight, and reproduction, as the change in total number of organisms, are used to measure the effect of a toxicant on life-cycle tests; data are analyzed statistically to indicate that concentration at which a significant difference occurs between the test solutions and control(s).

1.4 The results of dose-response acute or chronic toxicity tests with toxicants added experimentally to salt water should usually be reported in terms of an ~~LC50.~~ LC50 (mortality), or EC50 (medial effect concentration). The results of life-cycle toxicity tests with toxicants added experimentally to salt water should be reported as that concentration at which a statistically significant difference in the number of offspring or growth (determined by weight) is produced with reference to the control(s).

1.5 Where appropriate, this standard has been designed to be consistent with or complementary to other methods for assessing toxicity to invertebrates described in Test Methods E1367 and E1706, and Guides E1391, E1525, E1611, and E1688.

[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Actionand is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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1.6 This guide is arranged as follows:

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

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate ~~safety~~ safety, health, and ~~health~~environmental practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 7.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D4447 Guide for Disposal of Laboratory Chemicals and Samples
E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
E1367 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates
E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates
E1525 Guide for Designing Biological Tests with Sediments
E1611 Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids
E1688 Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates
E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates
E1733 Guide for Use of Lighting in Laboratory Testing
E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines (Withdrawn 2022)[3]
IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

3. Terminology

3.1 *Definitions:*

3.1.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to express the strongest possible recommendation, just short of an absolute requirement, that is, to state that this test ought to be designed to satisfy the specific condition, unless the purpose of the test requires a different design. "Must" is used only in connection with factors that relate directly to the acceptability of the test (see Section ~~13~~14). "Should" is used to state that the specific condition is recommended and ought to be met, if possible. Although 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."

3.1.2 For definitions of other terms used in this guide, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 The toxicity of a substance in marine or estuarine waters can be determined through a ~~96-h~~96 h acute or chronic toxicity test (see 11.1.2) in 100-mm diameter glass petri dishes for *Neanthes arenaceodentata* and *Capitella capitata*. Stender dishes are recommended for acute, chronic, and life-cycle tests with the smaller species *Ophryotrocha diadema* and *Dinophilus gyrociliatus*. Petri dishes provide ample horizontal space to minimize cannibalism in the case of *Neanthes arenaceodentata*. Larger containers, such as ½ gallon – 1 gallon jars, are necessary for life-cycle tests using *Neanthes arenaceodentata* and *Capitella capitata*. The static-renewal technique is recommended for the chronic and life-cycle tests with these latter species; it is usually not necessary to renew the solutions in 96-h tests. With either the static or static-renewal system, data on the concentration of test material are obtained and analyzed to determine the effect(s) of the toxicant on survival and reproduction.

5. Significance and Use

5.1 Polychaetes are an important component of the benthic community, in which they generally comprise 30 to 50 % of the macroinvertebrate population. They are preyed upon by many species of fish, birds, and larger invertebrate species. Larger polychaetes feed on small invertebrates, larval stages of invertebrates, and algae. Polychaetes are especially sensitive to inorganic

[2] 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.
[3] The last approved version of this historical standard is referenced on www.astm.org.

toxicants and, to a lesser extent, to organic toxicants (**1**).[4] The ecological importance of polychaetes and their wide geographical distribution, ability to be cultured in the laboratory, and sensitivity to contaminants make them appropriate acute and chronic toxicity test organisms. Their relatively short life cycle enables the investigator to measure the effect of contaminants on reproduction.

5.2 An acute toxicity or chronic text is conducted to obtain information concerning the immediate effects of an exposure to a test material on a test organism under specified experimental conditions. An acute toxicity test provides data on the short-term effects, which are useful for comparisons to other species but do not provide information on delayed effects. Chronic toxicity tests provide data on long-term effects.

5.3 A life-cycle toxicity test is conducted to determine the effects of the test material on survival, growth, and reproduction of the test species. Additional sublethal endpoints (for example, biochemical, physiological, and histopathological) may be used to determine the health of the species under field conditions.

5.4 The results of acute, chronic, and life-cycle toxicity tests can be used to predict effects likely to occur on marine organisms under field conditions.

5.5 The results of acute, chronic, or life-cycle toxicity tests might be used to compare the sensitivities of different species and the toxicities of different test materials, as well as to study the effects of various environmental factors on the results of such tests.

5.6 The results of acute, chronic, or life-cycle toxicity tests might be an important consideration when assessing the hazards of materials to marine organisms (see Guide E1023) or when deriving water quality criteria for aquatic organisms (**2**).

5.7 The results of acute, chronic, or life-cycle toxicity tests might be useful for studying the biological availability of, and structure activity relationships between, test materials.

5.8 The results of acute, chronic, and life-cycle toxicity tests will depend partly on the temperature, quality of food, condition of test organisms, test procedures, and other factors.

6. Apparatus

6.1 *Facilities*—Aquaria ~~(4~~(2 L to 57 L) or gallon jars containing either clean (uncontaminated), natural, or reconstituted sea water should be used for culturing these four species of polychaetes or holding them after field collection and prior to a test. Aquaria provided with aeration have been used successfully for culturing and holding polychaetes. The holding tanks and any area used for culturing or holding polychaetes should be located in a room or space separated from that in which toxicity tests are to be conducted. The sea water should be analyzed periodically to ensure a constant salinity. The holding aquaria, water supply, or room in which they are kept should be equipped with temperature control. Aeration can be provided to ensure that dissolved oxygen is greater than 60 % saturation and that water circulation is adequate in the aquaria. ~~These species of polychaetes do not require a definite light regime.~~A consistent light cycle including 12 h -16 h of light should be provided.

6.2 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or any water into which test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that affect the test organisms adversely. In addition, equipment and facilities that contact stock or test solutions should be chosen to minimize the sorption of test materials from water. Glass, Type 316 stainless steel, nylon, high-density polyethylene, ~~polycarbonate, and fluorocarbon~~ polycarbonate plastics should be used whenever possible to minimize dissolutions, leaching, and sorption, except that stainless steel should not be used in tests on metals in salt water. Fluorocarbon plastics (that is, per- and polyfluoroalkyl substances or PFAS) should be avoided in studies evaluating these types of chemicals. Concrete and rigid plastics may be used for holding tanks and in the water-supply system, but they should be soaked, preferably in flowing dilution water, for one week or more before use (**3**). Cast iron pipe should not be used with salt water and probably should not be used in a fresh water-supply filter system because colloidal iron will be added to the dilution water and strainers will be necessary to remove rust particles. A specially designed system is usually necessary to obtain salt water from a natural water source (**4**). Brass, copper, lead, galvanized metal, and natural rubber should not contact the dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber or other materials not mentioned previously should not be used unless it has been shown that their use will not affect either the survival, growth, or reproduction of polychaetes adversely.

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

6.3 *Test Chambers*—In a toxicity test with marine organisms, test chambers are defined as the smallest physical units between which there are no water connections. Glass petri dishes measuring 100 mm in diameter and 20 mm in height are the most satisfactory chambers for use in acute and chronic toxicity tests with *Neanthes arenaceodentata* and *Capitella capitata*. Only one *Neanthes arenaceodentata* should be placed in a single chamber since this species can be cannibalistic. Stender dishes measuring 40 mm in diameter and provided with a ground glass cover are the most satisfactory chambers for acute, chronic, and life-cycle tests with *Ophryotrocha diadema* and *Dinophilus gyrociliatus. Capitella capitata*,*Ophryotrocha diadema*, and *Dinophilus gyrociliatus* are not cannibalistic, and 5 to 10 specimens may be placed within a single chamber. For reproductive tests, ~~glass~~ chambers measuring 3.79 L and containing 2.5 L of test solution should be used with *N. arenaceodentata* and *C. capitata*. Aeration must be provided. Chambers the same size as that used in the acute and chronic tests with *O. diadema* and *D. gyrociliatus* can be used for the reproductive tests. The chambers should be covered to keep out extraneous contaminants and to reduce the evaporation of test solution and test material. All chambers in a test must be identical.

6.4 *Cleaning*—Test chambers and other glassware, and equipment used to store and prepare the test sea water, stock solutions, and test sediments, should be cleaned before use. New items should be cleaned before each use by washing with laboratory detergent and rinsing with water, a weak-miscible organic solvent, and acid (10 % nitric or hydrochloric acid), and they should be rinsed twice with distilled, deionized, or dilution water. Metals, sulfides, and carbonate deposits are removed by the acid rinse, and organics are removed by the organic solvent rinse. A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and acid rinses, but it might attack silicone adhesives. At the end of each test, all items that are to be used again should immediately be (*1*) emptied; (*2*) rinsed with water; (*3*) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and solvents to remove organics); and (*4*) rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits. The test chambers should be rinsed with dilution water just before use. Glassware used only for live animals, not exposed to toxicants, should be cleaned using only distilled or clean dilution water, since the use of detergents is sometimes detrimental to live organisms.

6.5 *Acceptability*—The acceptability of new holding or testing facilities should be demonstrated by conducting a "non-toxicant" test in which all test chambers contain dilution water. Survival of the test species will demonstrate whether the facilities, water, control, and handling techniques are adequate to result in acceptable (90 %) survival of control animals in the absence of toxicants.

7. Safety Precautions

7.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and their solutions should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands into test solutions), laboratory coats, aprons, and glasses. Special precautions, such as covering the test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**5**), recommended handling procedures (**6**), and the chemical and physical properties of test material should be studied before a test is begun. Special precautions might be necessary with radiolabeled test materials (**7**) and with test materials that are, or are suspected of being, carcinogenic.

7.2 The use of ground fault systems and leak detectors is recommended strongly to help prevent electrical shocks because salt water is a good conductor of electricity.

7.3 Although the disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. The removal or degradation of test material might be desirable before the disposal of stock and test solutions.

7.4 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

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

7.6 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 in a fume hood.

8. Dilution Water

8.1 *Requirements*—The dilution water, if needed, should (*1*) be available in adequate supply, (*2*) be acceptable to the test organisms, (*3*) be uniform in quality, and (*4*) not affect the test results unnecessarily.

8.1.1 The minimum requirement for acceptable water for use in acute toxicity tests is that healthy test organisms survive in it for the duration of holding and testing without showing signs of stress such as unusual behavior, changes in appearance, or death. The water in which the test organisms are held prior to the test should be uniform. The range of temperature and salinity encountered during the holding period must not affect the survival of the test organisms adversely. A better criterion for an acceptable dilution water is that the test species will survive, grow, and reproduce satisfactorily in it.

8.1.1.1 *Salt Water*—Water in which polychaetes will survive, grow, and reproduce satisfactorily in a life-cycle test is probably an acceptable dilution water for these tests.

8.1.2 The quality of the dilution water should be uniform so that the test organisms are cultured or acclimated, and the test should be conducted in water of the same quality. In salt water, the range of salinity should be less than 2 g/kg or 20 % of the average salinity, whichever is higher.

8.1.3 The dilution water should not affect the results of an acute test unnecessarily because of such things as sorption or complexation of the test material. Therefore, except as in accordance with 8.1.4, the concentration of both total organic carbon (TOC) and particulate matter should be below 5 mg/L.

8.1.4 If it is desired to study the effect of an environmental factor such as TOC, particulate matter, or dissolved oxygen on the results of an acute test, it will be necessary to use water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such water is used, it is important that adequate analyses be performed to characterize the water and that a comparable test be conducted in a more usual dilution water in order to facilitate the interpretation of results in the special water.

8.2 *Source:*

8.2.1 *Reconstituted Water*—Some reconstituted salt waters prepared from either reagent-grade chemicals or sea salts have been shown to be acceptable for life-cycle toxicity tests with polychaetes (**8**). It might be desirable to condition (age) reconstituted ~~sale~~salt water by aerating it for two or more days.

8.2.1.1 If reconstituted water is used for tests with saltwater species, the reconstituted water described in Table 1 should be used whenever possible. If desired, reconstituted water may be prepared using a commercial sea salt preparation. Commercial sea salt mixes should be "bioassay grade" (such as Crystal Sea® Marinemix Bioassay Laboratory Formula) without the addition of the metal chelating agent ethylenediaminetetraacetic acid (EDTA) or dechlorinators such as sodium thiuosulfate. These compounds are present in hobbyist salts and may mask the toxicity of environmental or spiked sediment samples. The reconstituted water should be used at a salinity of 34 g/kg and pH = 8.0 for tests with true marine stenohaline species, and at a salinity of 17 g/kg and pH = 7.7 with euryhaline species. Other salinities may be used for studying the effects of water quality on the results of toxicity tests.

TABLE 1 Reconstituted Salt Water (from Practice E729)

NOTE 1—Add the following reagent-grade[5] chemicals in the amounts and order listed to 890 mL of ~~water.~~ water (see 8.2.1.2). Each chemical must be dissolved before the next is added.[A]

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
$NaHCO_3$	200 mg

[A] If the resulting solution is diluted to 1 L, the salinity should be 34 ± 0.5 g/kg and the pH 8.0 ± 0.2. The desired test salinity is attained by dilution at the time of use. ~~The reconstituted salt water should be stripped of trace metals.~~

8.2.1.2 Reconstituted ~~water~~seawater is prepared by adding ~~a sea salt for~~ specified amounts of reagent-grade ~~chemicals~~salts to high-quality water with (*1*) conductivity ~~below 1~~approximately <1 µS ~~micromho/cm~~/cm and (*2*) either TOC below 5 ~~mg/L.~~ mg/L or chemical oxygen demand (COD) less than 5mg/L. Acceptable water can usually be prepared using properly operated deionization, distillation, or reverse osmosis units. Conductivity should be measured on each batch, and TOC or ~~chemical oxygen demand (COD)~~ COD should be measured at least twice per year and whenever significant changes might be expected. If the water is prepared from a surface water, TOC or COD should be measured on each batch. The reconstituted water should be aerated intensively before use. Problems have been encountered with some species in some fresh and salt reconstituted waters, but sometimes these problems have been overcome by aging the reconstituted water for one or more weeks.

8.2.2 *Natural Dilution Water:*

8.2.2.1 If a natural dilution water is used, it should be obtained from an uncontaminated, uniform quality source. ~~The quality of water from a well or spring is usually more uniform than that of surface water.~~ If surface water is used as a source of dilution water, the intake should be positioned to minimize fluctuations in quality (for example, approximately 1 m below the surface) and the possibility of contamination, as well as to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron.

8.2.2.2 If desired, the hardness, salinity, pH, etc. of a water may be adjusted by the addition of appropriate reagent-grade chemicals, sea salt, acid, base, distilled or deionized water, etc. When necessary, sea salt may be added to salt water to prevent excessive decreases in salinity (see 8.2.1.1) if the salt has been shown to cause no adverse effects on the test species at the concentration used.

8.2.3 Chlorinated water must never be used for dilution water in toxicity tests because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals **(9)**. Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite, and both are more reliable than carbon filters, especially for removing chloramines **(10)**. Some organic chloramines, however, react slowly with sodium bisulfite **(11)**. In addition to residual chlorine, municipal drinking water often contains unacceptably high concentrations of metals, and its quality is highly variable. Excessive concentrations of most metals can usually be removed with chelating resin **(12)**, but the use of a different dilution water might be preferable. If dechlorinated water is used as dilution water or in its preparation, during the test it must be shown that either (*1*) a polychaete species will survive, grow, and reproduce acceptably in it or (*2*) fresh samples of dilution water supplied at least three times each week on nonconsecutive days do not cause either of the following: (*1*) the test species of polychaete to show more signs of stress, such as discoloration, unusual behavior, or death, when held in a water that was not chlorinated and dechlorinated; or (*2*) the concentration of chlorine-produced oxidants to be below 7.5 µg/L **(9)**.

8.3 *Treatment:*

8.3.1 Dilution water should be well aerated by using air stones, surface aerators, or column aerators before the addition of test material. Adequate aeration will bring the concentration of dissolved oxygen and other gases into equilibrium with air, minimize oxygen demand and concentrations of volatiles, and stabilize pH. The concentration of dissolved oxygen in the dilution water should be between 90 and 100 % **(13)** to help ensure that dissolved oxygen concentrations in the test chambers are acceptable.

8.3.2 Salt water from a surface water source should be passed through a filter effective to 15 µm or less to remove parasites and larval stages of predators.

8.3.3 When necessary, sea salt may be added to prevent excessive decreases in salinity (see 8.2.1) if the salt has been shown to cause no adverse effects on the survival, growth, or reproduction of polychaetes at the concentration used.

8.4 *Characterization:*

8.4.1 The following items should be measured at least ~~twice per year~~ annually or (*1*) more often if such measurements have not been made ~~semi-annually~~ for at least two years or (*2*) if surface water is used: salinity, pH, particulate matter, TOC, organophosphorus pesticides, organic chlorine (or organochlorine pesticides and polychlorinated biphenyls (PCBs)), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, fluoride, iodide, nitrate, phosphate, sulfate, calcium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, tributyltin, and zinc.

8.4.2 For the purposes of 8.4.1, the term “organophosphorus pesticides” refers to ~~chloropyrifos,~~chlorpyrifos, demeton, diazinon,

disulfoton, fenitrothion, malathion, methyl parathion, and parathion; the term "organochlorine pesticides" refers to aldrin, chlordane, DDD, DDE, DDT, dieldrin, endosulfan, endrin, heptachlor, heptachlor epoxide, lindane, methoxychlor, mirex, and toxaphene; and the term "chlorinated phenoxy herbicides" refers to the free acids, salts, and esters of 2,4-D, dicamba, silvex, and 2,4,5-T. The term "organic chlorine" refers to chlorine that would be detected if, when samples are prepared for gas chromatographic analysis for polychlorinated biphenyls (PCBs) and the organochlorine pesticides listed above, a chlorine detector is used instead of an electron capture detector to measure compounds that elute from just before lindane to just after mirex on the gas chromatograph being used. Organic chlorine does not refer only to chlorine associated with organochlorine pesticides and PCBs; it also refers to all chlorine that elutes within the specified period.

8.4.3 The methods used should either (*1*) be accurate and precise enough to characterize the dilution water adequately or (*2*) have detection limits below concentrations that have been shown to affect estuarine and saltwater polychaetes adversely **(14)**.

9. Test Material

9.1 *General*—The test material should be reagent-grade[5] or better unless a test of formulation, commercial product, or technical-grade or use-grade material is specifically needed. The following should be known about the test material before a test is begun:

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than approximately 1 % of the material. Certificate of analysis (COA) provided by the manufacturer should be obtained and reviewed. The COA should remain on file.

9.1.2 Solubility and stability in dilution water.

9.1.3 Precision and bias of the analytical method at the planned test concentration(s) of the material.

9.1.4 Estimate of toxicity to humans.

9.1.5 Recommended handling procedures (see section 7.11).

9.1.6 Estimate of acute toxicity to test species.

9.2 *Stock Solution:*

9.2.1 In some cases, the test solution can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it and the dilution water should be determined before beginning the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is dilution water, although filtration or sterilization, or both, might be necessary. Deionized or distilled water may be used if the salinity of the dilution water will not be affected. Several techniques have been developed specifically for preparing aqueous stock solutions of slightly soluble materials **(15)**. The minimum necessary amount of strong acid or base may be used in the preparation of an aqueous stock solution, but such acid or base might affect the pH of test solutions appreciably. The use of a more soluble form of the test material, such as chloride or sulfate salt of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of the minimum necessary amounts of strong acids and bases.

9.2.3 If a solvent other than dilution water is used, its concentration in the test solutions should be kept to a minimum and should be low enough that it does not affect the survival of the polychaetes. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity to aquatic animals **(16)**, low volatility, and strong ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms, and acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[5] or better, and its concentration in any test solution should not exceed 0.5 mL/L. Higher concentrations may be used if shown through testing to not cause significant negative biological effects to the test organisms. A surfactant should not be used

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

in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient of a mixture, formulation, or commercial product unless an extra amount of solvent is used in preparation of the stock solution.)

9.2.4 If no solvent other than water is used, (*1*) a dilution water control must be included in the test and (*2*) the percentage of organisms in the control that show signs of disease or stress such as discoloration, unusual behavior, or death, must be 10 % or lower.

9.2.5 If a solvent other than water is used and the concentration of solvent is the same in all test solutions that contain test material, (*1*) at least one solvent control, containing the same concentration of solvent and using solvent from the same batch used to make the stock solution, must be included in the test, and (*2*) a dilution water control must be included in the test. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, ~~must~~should be 10 % or lower in the solvent control and ~~should~~must be 10 % or lower in the dilution water ~~control, if one is included in the test.~~control.

9.2.6 If a solvent other than water is used and the concentration of solvent is not the same in all test solutions that contain test material, both a solvent control, containing the highest concentration of solvent present in any other treatment and using solvent from the same batch used to make the stock solution, and a dilution water control must be included in the test. The percentage of organisms that show signs of disease or stress, such as discoloration, unusual behavior, or death, ~~must~~should be 10 % or lower in the solvent control and must be 10 % or lower in the dilution water control.

9.2.7 If a solvent other than water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning the possible effects of solvent on the test results.

9.3 *Test Concentration(s):*

9.3.1 If the test is intended to allow the calculation of an LC50, the test concentrations (see section 11.1.1.1) should bracket the predicted LC50. The prediction might be based on the results of a test on the same or a similar test material with the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which groups of five or more organisms are exposed for ~~24~~24 h to 96 h to a control and three to five concentrations of the test material that differ by a factor of ten. The greater the similarity between the range-finding test and the actual test, the more useful the range-finding test will be.

9.3.1.1 If the test is intended to be a life-cycle test to determine that concentration at which there is a statistically significant suppression in the number of young produced compared to the control, the results from an acute or chronic LC50 test can be used to predict the test concentration. The concentration at which there is a significant suppression of reproduction is generally between a factor of 2 to 10 times less than the 96 h LC50 for polychaetes (**1**).

9.3.1.2 If necessary, concentrations above solubility should be used because organisms in the real world are sometimes exposed to concentrations above solubility and because solubility in dilution water is often not well known. The use of concentrations more than ten times greater than solubility are probably not worthwhile. With some test materials, it might be found that concentrations above solubility do not kill or affect a greater percentage of test organisms than will the concentration that is the solubility limit; such information is certainly worth knowing.

9.3.2 In some (usually regulatory) situations, it is necessary to determine only (*1*) whether a specific concentration of test material is acutely toxic to the test species or (*2*) whether the LC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in the surface water, the concentration resulting from direct application of the material to a body of water, or the solubility limit of the material in water. When there is interest only in a specific concentration, it is often necessary to test only that concentration (see 11.1.5), and it is not necessary to actually determine the LC50.

10. Test Organisms

10.1 *Species*—Test species are usually selected on the basis of geographical distribution, availability, ease of handling in the laboratory, and past successful use. The appendixes of this guide provide information for the collection, identification, and culture of four species of polychaetes used in toxicity testing: *Neanthes arenaceodentata*,*Capitella capitata*,*Ophryotrocha diadema*, and *Dinophilus gyrociliatus*. Use of these species is encouraged in order to increase the comparability of results.

10.2 *Age*—The age of the test species suitable for each type of toxicity test is indicated in Table 2.

TABLE 2 Age of Test Species Suitable for Each Type of Toxicity Test

Species	Acute	Chronic	Life Cycle
~~*Neanthes arenaceodentata*~~	~~2 to 3 months[A]~~	~~1 to 3 months[A]~~	~~one month[A]~~
Neanthes arenaceodentata	2 weeks to 3 months[A]	2 weeks to 3 months[A]	one month[A]
Capitella capitata	2 to 3 weeks	2 to 3 weeks	1 day[A]
Ophryotrocha diadema	1 to 2 weeks	1 to 2 days	1 to 2 days
Dinophilus gyrociliatus	1 day	1 day	1 day

[A] Age is determined from the time of emergence from the parent's tube or egg capsule.

10.3 *Source*—It is generally more convenient to obtain specimens from a supplier rather than to use field-collected specimens. Cultures of ~~all four species are maintained by the Department of Biology, California State University, Long Beach, Long Beach, California. Cultures of~~ *Neanthes arenaceodentata* ~~is also maintained by the Environmental Division, U.S. Army Corps of Engineers, Vicksburg, Mississippi.~~ have been maintained by Aquatic Toxicology Support, LLC, Bremerton, Washington. Cultures of *Dinophilus gyrociliatus* ~~are~~have also been maintained by the U.S. Geological Survey, Texas. If field-collected specimens are to be used in testing, it is desirable to establish fresh cultures of *Capitella capitata*,*Ophryotrocha diadema*, or *Dinophilus gyrociliatus* because their short life cycle precludes a 14-day laboratory acclimation period. It is possible to hold *Neanthes arenaceodentata* in the laboratory for 14 days before testing because of its longer life cycle. Food, water, temperature, and salinity conditions for the laboratory stock and those held for an acclimation period must be similar to those conditions used in the toxicity test. All four species have been maintained and cultured successfully through many generations in the laboratory (**1**). The procedures for collecting, identifying, and culturing these species are given in Appendix X1 – Appendix X4.

10.3.1 If test organisms are cultured or held for an extended period of time in the laboratory, the response of laboratory-held organisms to contaminants should be compared to that of animals collected freshly from the field to ensure that apparent laboratory stresses do not affect their sensitivity to toxicants.

10.4 *Feeding*—It is not necessary to feed these species of polychaetes during an acute (96 h) toxicity test. It is necessary to feed these species during chronic and life-cycle tests. ~~Tetramarin®~~ Tetramin® or Tetramarine® fish flakes should be provided as the food source for maintaining and culturing these ~~species,~~ species (ground to <0.5 mm), as specified in the appendixes of this guide. During chronic or life-cycle tests, *Neanthes arenaceodentata* should be fed ground fish food flakes on an every-other-day basis at a rate of 8 mg (dry weight) per animal. Because *Capitella capitata*,*Ophryotrocha diadema*, and *Dinophilus gyrociliatus* are smaller than *N. arenaceodentata*, they are fed a mixture of 25 mg ground fish food flakes in 100-mL sea water. One or more drops of this suspension is added to each dish, as required. *Ophryotrocha diadema* and *Dinophilus gyrociliatus* can also be fed 50 µL of a 0.5 % spinach food suspension (w/v).

10.5 *Holding*—If the polychaetes are obtained from a supplier, they should be acclimated fully to the test temperature and salinity conditions prior to their use in a toxicity test. On the other hand, if the worms are being cultured by the laboratory conducting the test, the acclimation period is not necessary if the test is being conducted under the same environmental conditions.

10.6 *Quality*—All polychaetes used in a test must be in healthy condition (see 10.6.1). A qualified polychaete taxonomist must be consulted to ensure that the animals in the test population are all of the same species.

10.6.1 Polychaetes in holding containers should be checked daily before initiation of the test. Individuals that appear unhealthy or dead should be discarded. If more than 5 % of the polychaetes appear to be unhealthy during the 48 h preceding the test, the entire group should be discarded and not used in the test. It is necessary to examine *Capitella capitata*,*Ophryotrocha diadema*, and *Dinophilus gyrociliatus* under a dissecting microscope to determine the health of these species.

10.6.2 ~~Analysis of the test species~~It may be desirable to analyze field collected *Neanthes arenaceodentata* ~~and~~prior ~~Capitella capitata~~to testing ~~for the test material, if it~~ to determine if they have been exposed to the chemical being tested, if the chemical might be present in the ~~environment, and other chemicals to which exposure might have occurred is desirable. It will not be possible to analyze~~ environment. Pre-exposure to a chemical may affect the test result by making the test organisms either ~~Ophryotrocha diadema~~more or ~~Dinophilus gyrociliatus~~less sensitive. ~~for chemicals because of their small size.~~ Polychaetes may be used without chemical analysis if the specimens are obtained from an area that is monitored for chemical contamination and known to be free of toxicants and they are held in clean, uncontaminated water and facilities. It is not necessary to perform a

chemical analysis if the specimens are obtained from laboratory cultures that are cultured in clean, uncontaminated water and facilities. Polychaetes from contaminated areas should not be used in toxicity tests unless the experimental design specifically requires the use of that population.

10.6.3 The survival of polychaetes in control sea water during the test is an indication of the health of the population and other factors. The test must be considered invalid if a mean mortality above 10 % occurs in the controls in acute tests, or more than 20 % occurs in the controls of chronic or life cycle tests. A life-cycle test must be considered invalid if the animals fail to reproduce in the control.

10.6.4 Reference toxicants might be useful for monitoring the quality and sensitivity of the test organisms.

10.6.4.1 A reference toxicant can be useful for assessing the sensitivity of different populations or species of polychaetes, or seasonal variation in the sensitivity of field-collected populations. Such assessment is usually conducted simultaneously with the toxicity test. Many chemicals have been used or evaluated for use as reference toxicants (**17**). None has been proven to be a reliable indicator of the overall quality of any species or test results. A reference toxicant is likely to be more useful when used in conjunction with tests on materials that have the same mode of action as the reference toxicant. However, frequent changing among reference toxicants can reduce the value of reference toxicant data if there is not an adequate history of use with each procedure, species, and laboratory.

11. Procedure

11.1 *Experimental Design*—Decisions concerning aspects of experimental design, such as the dilution factor, number of treatments, and numbers of specimens and replicates, should be based on the purpose of the test and the type of procedure that is being used to calculate the results (see Section ~~14~~15). One of the following three types of experimental design will probably be appropriate in most cases.

11.1.1 *Acute Test*—An acute test is generally conducted for a 96 h period and is intended to measure the toxicity, as determined by survival or death, of a test material.

11.1.2 *Chronic Test*—A chronic test is conducted for a period of time greater than 96 h. ~~Survival and death~~ Survival, death, and growth (as weight) is used as the measure of toxicity. However, because of the short life cycle of *Dinophilus gyrociliatus*, the acute and chronic tests are the same. The chronic test with *Ophryotrocha diadema* can run for 10 days, for 14 to 21 days with *Capitella capitata*, and for up to 28 days with *Neanthes arenaceodentata*.

11.1.3 *Life-Cycle Test*—A life-cycle test begins with juvenile polychaetes and extends until they lay eggs. These four species lay their eggs in either the parent's mucoid tube or in a capsule, and all are fertilized at the time of laying (see Appendixes). The number of embryos laid per female is used as the measure of toxicity of the test material. The life-cycle test requires three months for *Neanthes arenaceodentata*, five weeks for *Capitella capitata*, four weeks for *Ophryotrocha diadema*, and ten days for *Dinophilus gyrociliatus*. Each of these tests can be extended to determine the viability of the embryos.

11.1.4 Acute and chronic tests intended to allow the calculation of an LC50 and life-cycle tests intended to measure the effect on reproduction usually consist of one or more control treatments and a geometric series of at least five concentrations of test material. In the dilution water or solvent control(s), or both (see 9.2.3 – 9.2.5), polychaetes are not exposed to test material. Except for the control(s) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.6, five properly chosen concentrations will often provide sufficient data for several durations ~~(see section 11.10.3)~~ and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low.

11.1.5 Although most toxicity tests use five test concentrations plus control(s), in some instances it might be necessary to determine only whether a specific concentration affects survival. If this is the case, only that concentration and the control(s) are necessary. Two additional concentrations at approximately one-half and two times the specific concentration of concern are desirable in order to increase confidence in the results.

11.1.6 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be assigned independently (**18**). In general, as the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom per treatment increases, and, therefore, the width of the confidence interval on a point estimate decreases, and the power of a hypothesis test increases. With respect to factors that might affect the results within test chambers and, therefore, the results of the

test, all chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. The test chambers are usually arranged in one or more rows. Treatments must be assigned randomly to individual test chamber locations and may be reassigned randomly during the test. A randomized block design (with each treatment being present in each block, which may be in a row or a rectangle) is preferable to a completely randomized design.

11.1.7 The effect of the test material on ~~survival~~ survival, growth, and reproduction cannot be determined accurately if any factor that affects one or more of them is too dissimilar between experimental units. Since the sex of most polychaetes cannot be determined until gametes begin to form, it may be impossible to determine whether any sexual difference exists in the effect of a particular toxicant. This is true for *Neanthes arenaceodentata*. The sexes of *Capitella capitata* can be distinguished by the presence of specialized genital hooks in the male (see Appendix X2). *Ophryotrocha diadema* is a protandric hermaphrodite, and only the females of *Dinophilus gyrociliatus* emerge from the egg capsule (see Appendix X3 and Appendix X4).

11.1.8 The minimum desirable number of test chambers and organisms per treatment should be calculated from (*1*) the expected variance within test chambers, (*2*) the expected variance between test chambers within a treatment, and (*3*) the maximum acceptable width of confidence interval **(18)**. If each test concentration is more than 60 % of the next higher one, fewer organisms per concentration of test material, but not the control treatment(s), may be used. If such calculations are not made, at least 10 organisms should be exposed to each treatment. Organisms in a treatment should be divided between two or more test chambers in order to permit the estimation of experimental error **(19)**. If the controls are important in the calculation of results, such as because of correction for spontaneous mortality using Abbott's formula, it might be desirable to use more test chambers and test organisms for the control treatment(s) than for each of the other treatments.

11.1.9 It is desirable to repeat the test at a later time to obtain information concerning the reproducibility of the results.

11.2 *Test Condition Specifications:*

11.2.1 *Dissolved Oxygen*—The concentration of dissolved oxygen in each test chamber must be from 60 to 100 % of saturation **(13)** during the entire test. It is not necessary to aerate the test chambers in the acute or chronic tests. It is necessary only to aerate *Neanthes arenaceodentata* and *Capitella capitata* during the life-cycle test. If provided, aeration should be the same in all test chambers, including the control(s), throughout the test. If aeration is used, it might be desirable to conduct a simultaneous test without aeration to determine aeration affects the test results.

11.2.2 *Temperature*—Tests with polychaetes should be conducted at 17 to 20°C. For each individual test chamber in which temperature is measured, the time-weighted average measured temperature at the end of the test should be within 1°C of the selected test temperature. The difference between the highest and lowest time-weighted averages for the individual test chambers must not be greater than 1°C. Temperatures must be within 3°C of the mean of the time-weighted averages. Whenever temperature is measured concurrently in more than one test chamber, the highest and lowest temperatures must not differ by more than ~~2°C.~~2 °C.

11.2.3 *Loading*—The amount of grams of organisms (whole body, wet weight, and blotted dry) per ~~litre~~liter of solution in the test chambers should not be so high that it affects the results of the test. Therefore, the loading should be limited to ensure that (*1*) the concentrations of dissolved oxygen and test material do not fall below acceptable levels, (*2*) concentrations of metabolic products do not exceed acceptable levels, and (*3*) the test organisms are not stressed because of aggression or crowding.

11.2.3.1 A smaller number of test organisms should be used if aggression occurs.

11.2.4 *Salinity*—The salinity in the toxicity tests must be within the tolerance range of the selected species of polychaetes. The optimum salinity is 30 g/kg to 35 g/kg for all species except *Dinophilus gyrociliatus*. The optimum salinity for this later species is 25 g/kg to 30 g/kg. If a test salinity other than the optimum salinity is used, an additional control at the optimum salinity must be employed.

11.2.5 *Light*—The light in the laboratory should be maintained on a ~~12-h~~12 h light, ~~12-h dark photoperiod.~~12 h dark or a 16 h light, 8 h dark photoperiod. (refer to Guides E1192, E1367, E1525, and E1733).

11.3 *Beginning the Test:*

11.3.1 The toxicity test begins when the test organisms are first placed in test chambers containing test material.