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

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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 from laboratory cultures and exposed to varying concentrations of a toxicant in static 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, oil drilling fluids, produced water from oil well production, and other types of industrial wastes. An LC50 (medial lethal concentration) is calculated from the data generated in each acute and chronic toxicity test. Reproductive success and the number of offspring produced 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 acute or chronic toxicity tests with toxicants added experimentally to salt water should usually be reported in terms of an LC50. 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 is produced with reference to the control(s).

1.5 This guide is arranged as follows:

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

1.7 *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 precautionary statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

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

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). "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 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 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 toxicants and, to a lesser extent, to organic toxicants (**1**).[3] 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 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.

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

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 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.

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 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. 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.

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 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. 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.

8.2.1.2 Reconstituted water is prepared by adding a sea salt for specified amounts of reagent-grade chemicals to high-quality water with (*1*) conductivity below 1 micromho/cm and (*2*) TOC below 5 mg/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) 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:*

TABLE 1 Reconstituted Salt Water (from Practice E729)

NOTE 1—Add the following reagent-grade[4] chemicals in the amounts and order listed to 890 mL of water. 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.

8.2.2.1 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 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, 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[4] 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.

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

[4] *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.

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[4] or better, and its concentration in any test solution should not exceed 0.5 mL/L. A surfactant should not be used 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 be 10 % or lower in the solvent control and should be 10 % or lower in the dilution water control, if one is included in the test.

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 be 10 % or lower in the solvent control and 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 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.

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. Cultures of *Dinophilus gyrociliatus* are also 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® should be provided as the food source for maintaining and culturing these species, as specified in the appendixes of this guide. During chronic or life-cycle tests, *Neanthes arenaceodentata* should be fed 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 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).

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]
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.

10.5 *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 *Neanthes arenaceodentata* and *Capitella capitata* for the test material, if it might be present in the environment, and other chemicals to which exposure might have occurred is desirable. It will not be possible to analyze either *Ophryotrocha diadema* or *Dinophilus gyrociliatus* 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). 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 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 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.

11.2.3 *Loading*—The amount of grams of organisms (whole body, wet weight, and blotted dry) per litre 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 to 35 g/kg for all species except *Dinophilus gyrociliatus*. The optimum salinity for this later species is 25 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 light, 12-h dark photoperiod.

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.

11.3.2 A representative sample of the test organism must be either (*1*) distributed randomly among the test chambers by adding to each chamber no more than 20 % of the number of test organisms to be placed in each chamber and repeating the process until each chamber contains the desired number of test organisms; or (*2*) assigned by random number of one organism to each chamber, by random assignment of a second organism to each chamber, or by total randomization. It might be convenient to assign organisms to other containers and then to add them to the test chamber all at once.

11.3.3 On the day that the toxicity test is initiated, a sufficient number of polychaetes should be removed from the holding facility at one time to provide approximately one-third more animals than is needed. Select a set of test chambers (one test chamber from each test concentration plus control(s)) to be processed together in order to avoid possible selective bias during loading. Polychaetes can be transferred using a glass pipette with the bore larger than the largest specimen and with a smooth tip. Polychaetes should be handled gently to avoid injuries.

11.3.4 Tests should be begun by placing test organisms in the chambers within 30 min after the test material was added to the dilution water.

11.3.5 *Acute Tests*—Glass petri dishes, 100 mm in diameter and 20 mm deep, should be the containers used for acute tests with *Neanthes arenaceodentata* and *Capitella capitata*. Stender dishes or smaller sized petri dishes are recommended for tests involving *Ophryotrocha diadema* and *Dinophilus gyrociliatus*.

11.3.6 *Chronic Tests*—The same size petri dishes are used for chronic tests as for acute tests (11.5.5). It is necessary to feed the test animals during chronic tests, as specified in 11.5.

11.3.7 *Life-Cycle Tests*—A 3.78 L jar should be used for life-cycle tests conducted with *N. arenaceodentata* and *Capitella capitata*, as outlined in Appendix X1 and Appendix X2. Stender dishes are recommended for the life-cycle tests with *Ophryotrocha diadema* and *Dinophilus gyrociliatus*, as outlined in Appendix X3 and Appendix X4. All species must be fed during the life-cycle tests, as specified in 11.5.

11.4 *Feeding*—It is not necessary to feed these species of polychaetes during a 96-h acute toxicity test. All species must be fed during a chronic or life-cycle test, as specified in 11.4.1 – 11.4.4, as well in Appendix X1 – Appendix X4.

11.4.1 *Neanthes arenaceodentata* should be fed every other day at the rate of 8 mg fish food flakes per worm for both the chronic and life-cycle tests. It is important to observe each chamber prior to feeding in order to make certain that there is no accumulation of uneaten food. Worms are frequently unable to feed in the high concentrations of a toxicant, and uneaten food can reduce water quality in the chamber.

11.4.2 *Capitella capitata* should be fed fish food flakes at the rate of 0.1 mg per worm at the beginning of the test and every 7 days for the duration of the experiment. It is important to observe each chamber prior to feeding in order to make certain that there is no accumulation of uneaten food, which can reduce the water quality.

11.4.3 *Ophryotrocha diadema* is fed fish food flakes at the beginning of the test and at 14 days at the rate of 2 or 3 drops from a Pasteur pipette of a mixture of 0.1 g of fish food flakes in 50 mL of sea water. If the test is extended beyond 28 days, the worms should be fed at 28 days at the same rate.

11.4.4 *Dinophilus gyrociliatus* is fed 0.5 mL of a 0.5 % solution of either fish food flakes or spinach, only at the beginning of the test.

11.5 *Duration of Test*—The test period is 96 h for the acute test for all species. The test duration is 7 to 10 days for a life-cycle test with *Dinophilus gyrociliatus*. The test period is 14 days for a chronic test and 30 to 35 days for the life-cycle test with *Capitella capitata* and *Ophryotrocha diadema*. The test period is 20 days for a chronic test and 2 to 3 months for the life-cycle test for *Neanthes arenaceodentata*.

11.6 *Biological Data:*

11.6.1 The criteria for death of polychaetes are opaque white coloration, immobility (especially the absence of movement of parapodia), and lack of reaction to gentle prodding. Dead polychaetes must be counted, recorded, and removed daily. Live animals must be counted at the beginning of the experiment and daily to account for cannibalism or death resulting from impingement on the sides of test compartments. Missing or impinged animals should be recorded as such.

11.6.2 Live test organisms should not be stressed in an attempt to determine whether they are dead, immobilized, or

otherwise affected. The prodding of organisms and movement of test chambers during testing should be done very gently.

11.6.3 The number of dead organisms in each test chamber should be counted every 24 h after the beginning of the test. Counts should be performed more often if the shape of the toxicity curve is to be defined. A suggested schedule is to count the number of dead and affected organisms in each chamber at 3, 6, 12, and 24 h after the beginning of the test and twice per day thereafter until the end of the test.

11.6.4 Dead organisms should be removed at least once every 24 h if it can be done without stressing live organisms.

11.6.5 All polychaetes used in a test should be destroyed at the end of the test.

11.7 *Other Measurements:*

11.7.1 *Water Quality*—When dilution water is used, its hardness, alkalinity, conductivity, and pH should be measured, and the measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable. If a saltwater dilution water is used, its salinity and pH should be measured. Measurements of ammonia, particulate matter, total dissolved gas, and TOC are desirable. The dissolved oxygen concentration must be measured in at least one chamber in each treatment at the beginning and end in the control and the high, medium, and low test concentrations as long as live organisms are present. The dissolved oxygen must be measured at least every 48 h during the life-cycle tests with *Neanthes arenaceodentata* and *Capitella capitata*. The pH should be measured in at least one test chamber in each treatment at the beginning and end of the test in the control and in the high, medium, and low concentrations of the test material.

11.7.2 *Temperature:*

11.7.2.1 Throughout acclimation, temperature should be measured at least hourly, or the maximum and minimum temperatures should be measured daily.

11.7.2.2 In all tests, either (*1*) in at least one test chamber the temperature must be measured or monitored at least hourly, or the maximum and minimum temperatures must be measured daily; or (*2*) if the test chambers are in a water bath or a constant-temperature room or incubator, the temperature of the water or air must be measured at least hourly, or the maximum and minimum temperature must be measured at least daily. In addition, temperature must be measured concurrently near both the beginning and end of the test in all test chambers or in various parts of the water bath, room, or incubator.

11.7.3 *Test Material:*

11.7.3.1 If the test material is dispersed uniformly throughout the test chamber, water samples should be taken by pipetting through a glass tube from a point midway between the top, bottom, and side of the test chamber and should not include any surface material. If test material might be lost due to sorption onto the walls of the sample container, the container and the siphon or pipette should be rinsed in the test solution before collecting the sample. Water samples should be collected directly into containers of appropriate size, from which the test material can be extracted or analyzed directly. If the test material is not dispersed uniformly in the test chamber, the whole volume of the solution in the test chamber should be (*1*) used as a sample or (*2*) treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to distribute the test material uniformly before a sample is taken.

11.7.3.2 If some of the test material is not dissolved, measurement of the concentration of dissolved test material in the treatment might be desirable.

11.7.3.3 If possible, the concentration of test material should be measured in at least the control and high, medium, and low concentrations of test material at the beginning of the test (see 14.1). The measurement of degradation products might be desirable.

12. Analytical Methodology

12.1 If samples of the dilution water stock solution or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(19)** to minimize the loss of test material by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption and volatilization.

12.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sufficiently sensitive, methods should be obtained from other reliable sources **(20)**. The concentration of nonionized ammonia may be calculated from pH, temperature, and concentration of total ammonia **(21)**.

12.3 Methods used to analyze food (see 10.5) or test organisms (see 10.8) should be obtained from appropriate sources **(22)**.

12.4 The analytical method used to measure the concentration of toxicant in test chambers must be validated before beginning the test. The bias and precision of the method in an appropriate matrix should be determined whenever samples are analyzed using reference samples, reagent blanks, split samples, spiked recoveries, interlaboratory comparisons, or alternative methods of analysis, when appropriate.

12.5 In addition to measuring the total concentration of the toxicant in the water from test chambers, measurement of either the“ dissolved” fraction or “undissolved” fraction of the toxicant is desirable. The “dissolved” fraction is usually defined and determined as that which passes through a 0.45-µm membrane filter.

13. Acceptability of Test

13.1 The acute, chronic, or life-cycle tests should usually be considered unacceptable if one or more of the following occurred, except, for example, that if temperature was measured numerous times, a deviation of more than 3°C (see 13.1.11) in any one measurement might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if the temperature was measured more often.

13.1.1 All test chambers and compartments were not identical.

13.1.2 Treatments were not assigned randomly to individual test chamber locations.

13.1.3 A required dilution water or solvent control was not included in the test.

13.1.4 All animals in the test population were not from the same location or culture.

13.1.5 Individual test organisms were not assigned impartially or randomly to test chambers or compartments.

13.1.6 More than 10 % of the organisms in any required control treatment died during the acute test or more than 20% died during the chronic or life cycle test.

13.1.7 Dissolved oxygen and temperature were not measured as specified in 11.11.

13.1.8 Any measured dissolved oxygen concentration was not between 60 and 100 % of saturation in a test.

13.1.9 The difference between the time-weighted average measured temperatures for any two test chambers was greater than 1°C.

13.1.10 Any individual measured temperature in any test chamber was more than 3°C different from the mean of the time-weighted average measured temperatures for the individual test chambers.

13.1.11 The difference between the measured temperatures in any two test chambers was more than 2°C at any one time.

13.1.12 The salinity deviated beyond the optimum salinity of 30 to 35 g/kg for *Neanthes arenaceodentata*, *Capitella capitata*, and *Ophryotrocha diadema* and 25 to 35 g/kg for *Dinophilus gyrociliatus* at any one time.

13.1.13 Cultured and newly caught worms differed substantially in response (see 10.3.1).

13.2 The calculations of an LC50 should usually be considered unacceptable if either or both of the following occurred:

13.2.1 No treatment other than a control treatment killed or affected less than 37 % of the polychaetes exposed to it.

13.2.2 No treatment killed or affected more than 63 % of the polychaetes exposed to it.

14. Calculation of Results

14.1 *Acute and Chronic Tests:*

14.1.1 The LC50 and its 95 % confidence limits should be calculated on the basis of (*1*) the measured initial concentrations of the test material, if available, or the calculated initial concentrations for acute and chronic tests; and (*2*) the average measured concentrations of test material, if available.

14.1.2 Most acute and chronic toxicity tests produce quantal data, that is, counts of the number of alive or dead. A variety of methods **(23)** can be used to calculate an LC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed and contains two or more concentrations at which the percent dead is between zero and 100, but the most widely used are the probit, moving average, Spearman-Karber, and Litchfield-Wilcoxon methods. The method used should appropriately take into account the number of test chambers per treatment and the number of test animals per chamber. The binomial test can usually be used to obtain statistically reliable information on the LC50 even when fewer than two concentrations kill between zero and 100 %. The binomial method does not provide a point estimate of the LC50, but it does provide a range within which the LC50 should lie. If desired, an interpolation procedure may be used to obtain an approximate LC50.

14.2 *Life-Cycle Tests:*

14.2.1 The primary data to be analyzed from a life-cycle test with polychaetes are those on (*1*) the survival of the parent generation, (*2*) the number of eggs or embryos produced by each female, (*3*) the survival of the embryos, and (*4*) the concentration of test material in the test solution in each treatment. No special treatment is required for males and females.

14.2.2 The number of alive and dead specimens of the parent generation in each chamber can be used to calculate the LC50 as indicated in 14.1.2.

14.2.3 The number of individuals of *Ophryotrocha diadema* or *Dinophilus gyrociliatus* in each compartment of each treatment are counted and compared to the control using the nonparametric Mann-Whitney Test **(23)**. Both *Neanthes arenaceodentata* and *Capitella capitata* lay their eggs, which are incubated by a parent, in a tube (Appendix X1 and Appendix X2). The number of eggs laid within each tube can be counted under a dissecting microscope. The number of eggs laid per female within a test concentration is compared to the control using the non-parametric Mann-Whitney Test **(23)**. Data from the life-cycle tests are used to determine that concentration at which there is a suppression of reproduction.

14.3 The precision of the toxicity test is dependent on the number of replicates, number of individuals, and variability of the effect among replicates. Toxicity tests with polychaetes usually use five test concentrations plus control(s), with two or three replicates of 20 polychaetes per replicate. Increasing the number of replicates per test concentration might improve the precision of the toxicity test. For example, four replicates of 10 polychaetes each rather than two replicates of 20 polychaetes each might increase the precision of the test.

14.4 An LC near an extreme of toxicity, such as an LC5 or LC95, should not be calculated unless at least one concentration of test material killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the LC is to be calculated. Other ways of providing information concerning the extremes of toxicity are (*1*) to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment(s) or (*2*) to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are usually more reliable than reporting a calculated result such as an LC5 or LC95 unless several percent killed or affected were obtained close to 5 or 95 %.

14.5 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material killed or affected a statistically significant number of the exposed organisms. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and heterogeneity tests. A pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should then be used. The presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, minimum detectable difference, and power of the test.

15. Report

15.1 Include the following information, either directly or by reference to other available documents, in the record of results of an acceptable acute toxicity test:

15.1.1 Name of test and investigator(s), name and location of laboratory, and dates of initiation and termination of the test.

15.1.2 Source of test material, its 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.

15.1.3 Source of the dilution water, its chemical characteristics and a description of any pretreatment, and results of any demonstration of the acceptability of the water to an aquatic species.

15.1.4 Source of brood stock, place and date of collection (if from a wild population) of the test organisms, scientific name, name of person who identified the organisms and the taxonomic key used, observed diseases or unusual appearance, treatments, holding and acclimation procedures, and age of the polychaetes at the beginning of the test.

15.1.5 Description of the experimental design, test chambers and covers, depth and volume of the solution in the chambers, temperature, salinity, lighting, method of beginning the test, and number of polychaetes and chambers used per treatment.

15.1.6 Average and range of the measured concentration of dissolved oxygen (as percent of saturation) for each treatment and a description of any aeration performed on test solutions before or during the test.

15.1.7 Averages and ranges of the acclimation and test temperatures and method(s) of measuring or monitoring, or both.

15.1.8 Schedule for obtaining samples of test solutions and methods used to obtain, prepare, and store the samples.

15.1.9 Methods used for and results (with standard deviations or confidence limits) of chemical analysis of the water quality and concentration(s) of test material, impurities, and reaction with degradation products, including validation studies on other effects.

15.1.10 Definition of the effect(s) used for calculating EC50s and a summary of general observations on other effects.

15.1.11 Table of data on the number of test organisms exposed and killed at various times throughout the test in each test chamber in each treatment, including the control(s), in sufficient detail to allow independent statistical analyses.

15.1.12 The 24-, 48-, and 96-h LC50s, and their 95 % confidence limits, and the method used to calculate them; highest concentration of test material that killed or affected no greater a percentage of the test organisms than did the control treatment. Specification of whether the results are based on measured or unmeasured concentrations of the test material. For formulations and commercial products, specification of whether the results are based on whole mixture or active ingredient.

15.1.13 Anything unusual concerning the test, any deviation from these procedures, and any other relevant information.

15.2 Include enough information in published reports to identify the procedures used and quality of the results clearly.

16. Keywords

16.1 acute toxicity tests; aquatic tests; *Capitella capitata*; chronic toxicity tests; *Dinophilus gyrociliatus*; estuarine environments; experimental design; exposure tests; LC50 tests; life-cycle toxicity tests; marine environments; *Neanthes arenaceodentata*; *Ophryotrocha diadema*; polychaetes; reproductive tests; saline water; static tests; toxicity; toxicology

APPENDIXES

(Nonmandatory Information)

X1. *NEANTHES ARENACEODENTATA*—LIFE-CYCLE CULTURE TECHNIQUES

X1.1 *Introduction—Neanthes arenaceodentata* is widely distributed throughout the world; it has been collected in New England, Florida, California, Baja California, Hawaii, Central Pacific, and Europe, primarily from estuarine intertidal sand or muddy sand beaches (**1**). Population densities as high as 1000/m^2 have been observed. The polychaete lives in the sediment, where it constructs non-permanent mucoid tubes that consist of sediment, food, and fecal material. Specimens will measure up to 10 cm in length and are tan to yellow in color. Since many members of the family Nereidae are similar in size, color, and appearance, *N. arenaceodentata* can be distinguished from the other species by the possession of a continuous ring of paragnaths around Areas V–VIII of the everted proboscis and by a hooked tip present on the blade of the neuropodial heterogomph seta (Fig. X1.1 and Fig. X1.2). It is necessary to kill the animal in order to see these distinguishing

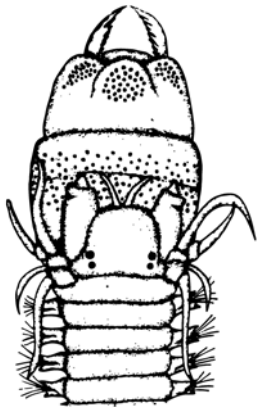

FIG. X1.1 *Neanthes arenaceodentata,* Anterior End With Everted Proboscis Covered With Paragnaths

FIG. X1.2 *Neanthes arenaceodentata*, Neuropodial Heterogomph Falcigerous Seta With a Hook at the Tip

characters. A laboratory population of this species has been maintained by the department of Biology, California State University, Long Beach, Long Beach, California, since 1964. Cultures of this species are also maintained by the Environmental Division, U.S. Army Corps of Engineers, Vicksburg, Mississippi, and by the Marine Laboratory, U.S. Environmental Protection Agency, Narragansett, Rhode Island. It may be more convenient to obtain laboratory-reared specimens from one of these laboratories rather than to obtain them from field collections. A difference in the chromosome numbers has been reported between the laboratory population originally collected from Southern California and specimens collected from the field in Connecticut and Hawaii. Thus far, the Connecticut and Hawaii populations have not been used as a test organism for toxicological studies.

X1.2 *Feeding Requirements—N. arenaceodentata* has been cultured through its complete life cycle using a variety of foods. Pesch and Schauer (**24**) made a comparison of the different foods used to culture this polychaete. They found fish food flakes to provide consistent growth throughout the life cycle. The water can be fouled easily with fish food flakes if the culture is overfed. Other foods that have been used to culture *N. arenaceodentata* include powdered alfalfa flour, powdered green algae (*Enteromorpha* spp. or *Ulva* spp.), or commercial rabbit food. Strands of *Enteromorpha* spp. are good for feeding and nest building for a reproductive pair (see X1.3.2).

X1.3 *Culture Techniques for Neanthes arenaceodentata:*

X1.3.1 *Juvenile and Adults Stage*—Stock colonies can be maintained at room temperature in aquaria of approximately 37 to 57 L in size with a maximum population of 75 to 100 worms. Sediment is not required to culture this species. Two air stones at opposite sides of the aquarium provide an adequate dissolved oxygen supply. Approximately 1.5 g of dried food should be provided once a week. The food must be soaked in sea water prior to feeding. The amount of food provided will vary according to the size of the worms, and whether the worms are getting too much or too little food should be determined prior to feeding. Overfeeding usually leads to the growth of mold or fungus on the bottom of the aquarium. The water level of the aquarium should be checked periodically and distilled water added to replace that lost by evaporation. Nearly all the water in the aquarium should be changed once per month. To remove worms from the aquarium for an experiment or other purposes, gather the clumps of *N. arenaceodentata* and food material with a fine net from the bottom and place them in a white pan with approximately 1 cm of sea water. The worms will free themselves from the tube masses, and they can be picked up with a pipette or fine art brush.

X1.3.2 *Reproductive Stage*—It is impossible to distinguish immature males from females on the basis of morphology. However, a behavioral difference occurs that can be used to distinguish the sexes (**1**). Males will fight males, and females will fight females. Fighting consists of everting their proboscis and extending their two jaws to grasp the other worm (Fig. X1.3). They can be cannibalistic. A male and female placed together will come alongside each other and lie side by side. A maturing female can be determined by observing yellow-orange ova maturing in her coelom. Place a female with developing ova in a petri dish with a sexually unknown worm and observe their behavior when they come into contact with one another. If they fight, the two worms are both females; if they lie side by side, the unknown worm is a male, and the pair can be used for reproduction. Place the pair in a 3.78-L jar containing 2.5 L of sea water, an air supply, and some soaked *Enteromorpha* spp., which will provide food and material for tube construction.

X1.3.2.1 The mature ova of *N. arenaceodentata* measure 0.5 to 0.6 mm in diameter. The eggs are probably released through breaks in the body wall between successive parapodia. After laying her eggs, the female dies within two to three days or can be eaten by the male. Fertilization has never been observed, but it is probably external. The fertilized eggs are clumped in the central part of the tube around the mid-body region of the male.

X1.3.3 *Embryo and Larval Stages*—The early stages of development through the 18 to 21 segmented stage proceeds within the tube of the male. The male circulates water through the tube with his body undulations. *N. arenaceodentata*, with its lecithotrophic egg, lacks a free-swimming trochophore larval stage characteristic of most polychaetes. The larvae do not feed but rather use the yolk as a source of energy. The larval body shape is distorted as a result of the yolk reserves (Fig. X1.4 and Fig. X1.5). When the yolk supply is depleted at

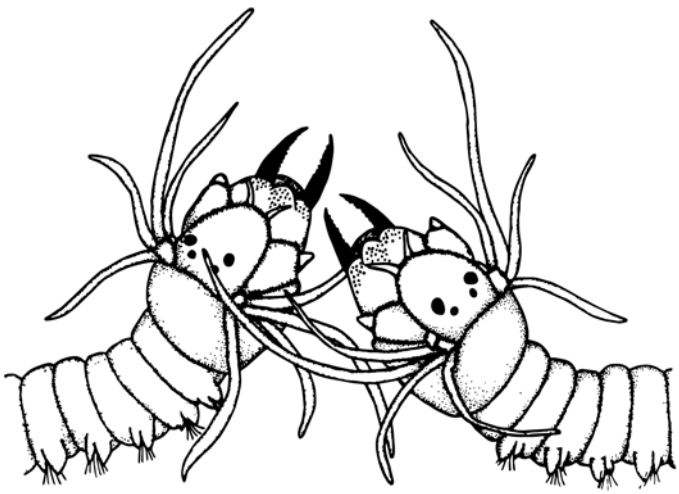

FIG. X1.3 *Neanthes arenaceodentata* of the Same Sex in Fighting Position

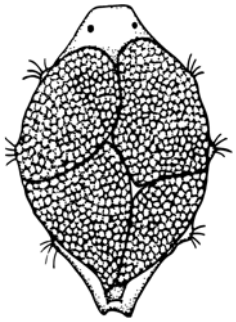

FIG. X1.4 *Neanthes arenaceodentata*, Three Segmented Larva

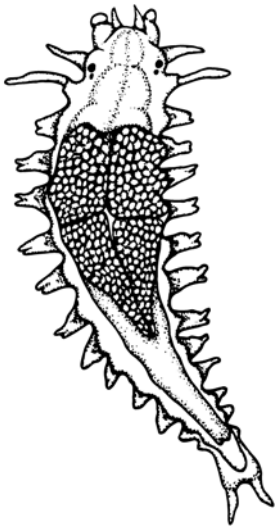

FIG. X1.5 *Neanthes arenaceodentata*, 12 Segmented Stage

approximately the 18 to 21 segmented stage, the young worm leaves the tube and commences feeding (Fig. X1.6). The early development within the male's tube generally takes three weeks under laboratory conditions (**25**). As soon as the young are observed leaving the tube, the juvenile worms should be placed in an aquarium and handled as indicated in X1.3.2. The male needs to be fed while the larvae develop since he may eat the larvae otherwise.

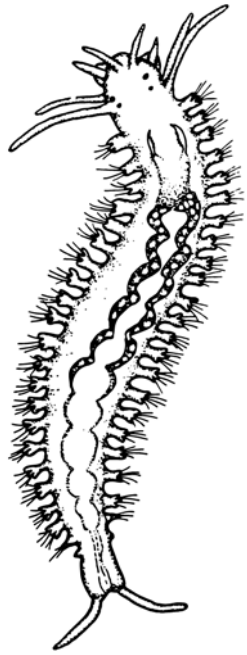

FIG. X1.6 *Neanthes arenaceodentata*, Juvenile, With 21 Segments and Has Just Left the Parent's Tube and Commenced Feeding

X2. *CAPITELLA CAPITATA*—LIFE-CYCLE CULTURE TECHNIQUES

X2.1 *Introduction—Capitella capitata* is a cosmopolitan species that is generally found in estuarine waters. It has been described as a non-competitive or opportunistic species because it flourishes in the absence of other polychaete species. *Capitella* is found in large numbers in the benthos in the vicinity of domestic sewage outfalls. This occurrence suggests that it is generally a pollution-tolerant species (**26**). However, tolerance of conditions around sewage outfalls does not necessarily indicate tolerance of all chemical pollutants. Specimens generally measure less than 2 cm in length, although larger individuals occur, especially in the vicinity of domestic sewage outfalls. Healthy specimens are bright red in color, and unhealthy or old specimens are dark red to brown in color. Grassle and Grassle (**27**) have described sibling species of *Capitella* that are distinguished by the morphology of the anterior and posterior ends, arrangement of teeth on the hooded hooks, weight of the specimens, method of reproduction, diameter of the eggs, number of eggs laid, and length of larval life. Using their sibling species classification, Type 2 has been the sibling species used in environmental studies (**28**) and the type whose culture techniques are described herein. Cultures of *Capitella capitata* have been maintained since 1968 by the Department of Biology, California State University, Long Beach, Long Beach, California.

X2.2 *Feeding Requirements*—Both natural and commercial foods have been used to feed *Capitella*, but fish food flakes is perhaps the most convenient. Stock colonies of adults can be maintained in 3.78-L aquaria (1-gal jars). Approximately 0.1 g of finely ground fish food flakes should be added to each aquarium each week; however, the powdered food must be mixed with seawater prior to feeding.

X2.3 *Culture Techniques for Capitella capitata*—Stock colonies of *Capitella* can be maintained at room temperature in 3.78-L aquaria provided with 2.5 L of filtered seawater and an air stone connected to an air supply. It is not necessary to provide a substrate; the food provides sufficient substrate for this species. It is not necessary to change the water; however, the entire colony is usually divided and placed in two or more aquaria every 3 to 4 weeks. Placing the colony in clean aquaria must be done to prevent deterioration of the colony within the aquarium after 6 to 8 weeks. New colonies can be established from trochophore larvae, as described below, but this is generally not necessary. Various stages in the life cycle are present within an aquarium.

X2.3.1 *Reproductive Stage—Capitella* is one of the few species of polychaetes that exhibit sexual dimorphism. Specialized genital hooks appear on the mid-dorsal region of setigerous segments 8 and 9 in males (Fig. X2.1); females lack these specialized setae (Fig. X2.2). Males may be sexed easily by observing the anterior region under a dissecting microscope and checking for the presence or absence of these genital hooks. Developing ova appear as white masses along the ventrum from segment 10 to 12 to the posterior end; this is the most convenient way to sex females. Copulation occurs, but it is rarely observed. Sperm are transferred to the female, with fertilization occurring either internally or externally at the time of egg discharge. The eggs are laid within the tube of the female (Fig. X2.3). The tube is a loosely constructed structure consisting of mucus, food, and fecal material. The female circulates water through the tube with her body undulations. The fertilized egg measures approximately 0.25 mm in diameter, and the color changes gradually from white to gray-green as development proceeds during the next 4 to 6 days. No special care of developing embryos is required in stock colonies.

X2.3.2 *Trochophore and Metatrochophore Stages*—The trochophore stage is reached approximately 4 to 6 days after egg-laying (Fig. X2.4). This stage is capable of moving freely within the tube either by ciliary movement or by contraction of longitudinal muscles. The trochophore may either swim free of the tube and become planktonic or proceed directly into the metatrochophore stage within the female's tube (Fig. X2.5). If it occurs, the planktonic trochophore of this sibling species is of short duration, and it soon settles to the substrate and develops into the metatrochophore stage. The trochophore and metatrochophore stages last 1 to 2 days before resembling a juvenile worm (Fig. X2.6). Growth is rapid under laboratory conditions, with egg masses beginning to develop in the coelom of a young female in approximately 20 days. The life cycle is completed in 25 to 40 days. Both males and females are capable of reproducing more than once.

X2.3.3 *Establishing New Stock Colonies from Larvae*—Occasionally, it may be necessary to establish new stock colonies of *Capitella*, especially if the exact age of the specimens is required. Examine a clump of worms from a stock colony that was established approximately 3 weeks earlier under a dissecting microscope. Look for females incubating embryos (Fig. X2.3). If the embryos appear gray-green in color, remove the entire tube and place it in a separate container. Tease the tube apart with forceps, which will free the trochophore-metatrochophore larvae to swim freely in the container. Pipette approximately 50 larvae into a separate dish, and then place the contents in a 3.78-L aquarium and feed as indicated in X2.2 and X2.3. The feeding schedule and reestablishment of clean aquaria is the same as described in X2.3.

X2.4 Abnormal larvae may develop in life-cycle tests at higher concentrations of a toxicant (**1, 29**). The most common abnormality is a two-tailed trochophore larva (Fig. X2.7). In life-cycle tests, it may be useful to check the embryos while still present in the tube. Abnormal larvae are best observed just prior to emergence using the procedures outlined in X2.3.3.

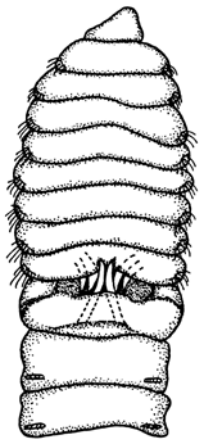

FIG. X2.1 *Capitella capitata*, Male, Dorsal View of Anterior End Showing the Genital Hooks in Setigerous Segments 8 and 9

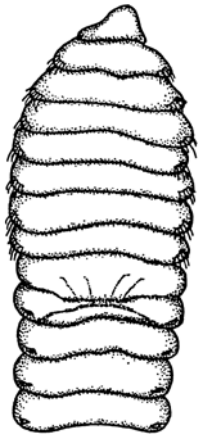

FIG. X2.2 *Capitella capitata*, Female, Dorsal View of Anterior End

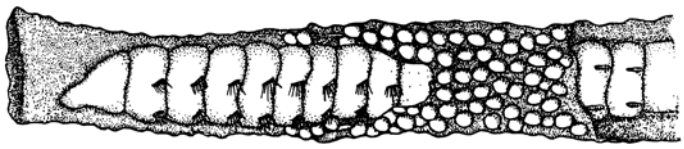

FIG. X2.3 *Capitella capitata*, Female Incubating Developing Embryos; the Embryos are Located Within the Mucoid Tube of the Female

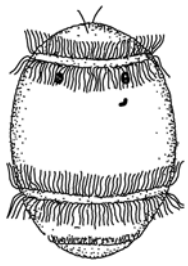

FIG. X2.4 *Capitella capitata*, Trochophore Larval Stage

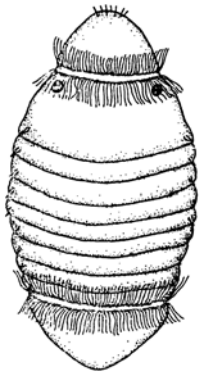

FIG. X2.5 *Capitella capitata*, Metatrophore Larval Stage

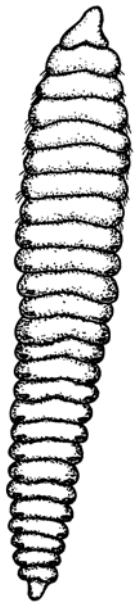

FIG. X2.6 *Capitella capitata*, Juvenile Worm

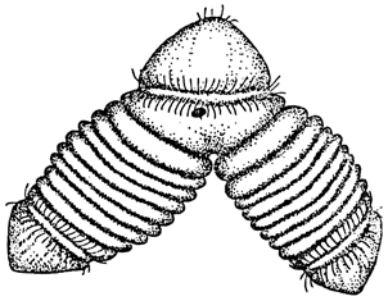

FIG. X2.7 *Capitella capitata*, Bifurcate Abnormal Larvae Induced by Sublethal Amounts of a Toxicant

X3. *OPHRYOTROCHA DIADEMA*—LIFE-CYCLE CULTURE TECHNIQUES

X3.1 *Introduction—Ophryotrocha diadema* was originally known from only two specimens collected from Los Angeles Harbor in 1972 (**30**). More recently, additionally specimens have been collected from the harbor as well as from Long Beach Marina. No one has attempted to collect them elsewhere from Pacific Coast marine waters. The original two specimens were taken to Goteborg, Sweden, where cultures are maintained by Bertil Akesson. Subcultures from the original two specimens have been shipped to different laboratories in the United States and Europe, where they have been used in environmental studies. Additional cultures have been established from specimens collected in 1989 and 1990. *Ophryotrocha diadema*is a minute species; adults measure up to 4.6 mm in length (Fig. X3.1). Species of the genus *Ophryotrocha* are very difficult to identify. *Ophryotrocha diadema* is known to co-occur with *O. labronica pacifica* and an undescribed species (Akesson, personal communication). They are distinguished by head pigmentation, method of reproduction, egg size, and larvae. The collecting technique is described below; however, for practical reasons, it would be more convenient to obtain a subculture from some other laboratory, for example, the University of Gothenborg or the Department of Biology, California State University, Long Beach.

X3.2 *Collecting Techniques*—Fouling communities attached to floating boat docks or pilings in protected waters should be collected and placed in a bucket containing seawater. The material should be swished around vigorously in the bucket; larger animals, such as mussels and tunicates, should be removed during this procedure. Pass the remaining material in the bucket through a 5.0-mm sieve. Discard the material retained on the sieve. Approximately 500 to 700 mL of the material that passed through the sieve is placed in a 2-L graduated cylinder. The cylinder is filled to near the top with clean sea water. The vessel is sealed with an air-tight stopper and placed in a dark place at room temperature for 4 to 8 h. As the dissolved oxygen is depleted, *Ophryotrocha diadema*, as well as other animals, migrate to the top of the cylinder. Species of *Ophryotrocha* can be identified by their black jaws, which can be seen with the aid of a hand lens. Pipette them into a petri dish containing filtered seawater. Since specimens may be injured during this process, do not place more than five specimens in a petri dish. Only 20 to 30 specimens are needed to establish a culture. Examine the animals each day, and remove any dead specimens; do not feed until 24 to 48 h after collection.

X3.3 *Feeding Requirements*—Frozen spinach, fish food flakes, and different green algal species have been used as food for *Ophryotrocha*, and they are all prepared in the same manner. The food is placed in a blender and chopped finely. The finely ground material is allowed to settle in the blender for approximately 5 min, at which time the supernatant are poured into a container and used as food. A few drops of the supernatant are pipetted into the dish containing animals; the amount of food given will depend on the population size. If the population is maintained at room temperature, feed only once per week.

X3.4 *Culture Techniques for Ophryotrocha diadema*—The life cycle of this species of *Ophryotrocha* is short, requiring 25 to 35 days for a complete life-cycle (**1, 30**). The culture techniques described are the same for all stages in the life cycle. *Ophryotrocha diadema* belongs to that group of *Ophryotrocha* species that is protandric hermaphrodites. It has a restricted number of anterior male segments and a restricted number of posterior female segments. Presumably, self-fertilization does not occur. Eggs are laid in linear, mucoid capsules (Fig. X3.2), where early development occurs to the 4 setigerous segmented stage (Figs. X3.3-X3.5). Stock colonies are cultured in 3.78-L aquaria. Each aquarium is filled with 2.5 L of filtered (preferably through a microporous filter system) seawater. Approximately 40 to 50 specimens are placed in each aquarium and fed as previously indicated. Aeration is provided with an air stone. The stock colony should be fed once per week. The population reaches its peak numbers in 5 to 6 weeks and then declines. A new stock culture is established by emptying the contents of one or more aquaria into a dark pan. Animals are pipetted into separate groups of 40 to 50 specimens, and a new stock culture is started as previously described.

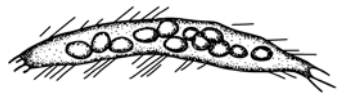

FIG. X3.2 *Ophryotrocha diadema*, Developing Embryos in a Mucoid Capsule

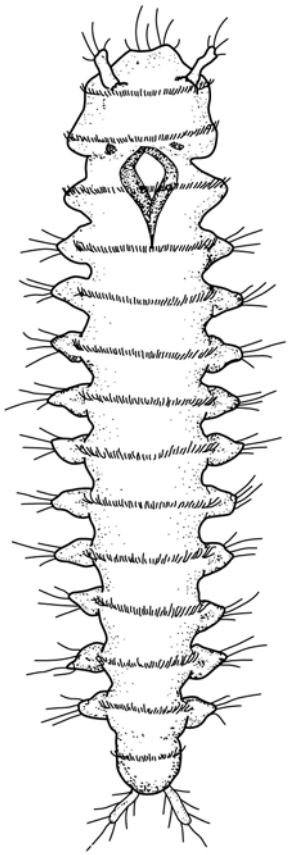

FIG. X3.1 *Ophryotrocha diadema*, Dorsal View of Adult Worm

FIG. X3.3 *Ophryotrocha diadema*, Larva From Egg Mass

FIG. X3.4 *Ophryotrocha diadema*, Larva From Egg Mass

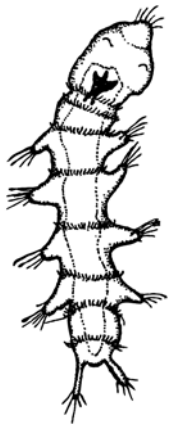

FIG. X3.5 *Ophryotrocha diadema*, Newly Released Larva From Egg Mass with Four Setigerous Segments

X4. *DINOPHILUS GYROCILIATUS* —LIFE-CYCLE CULTURE TECHNIQUES

X4.1 *Introduction—Dinophilus gyrociliatus* is a minute (0.5 to 1.0 mm) archiannelid polychaete that is known from widespread areas of the world. It is typically found in established aquaria at marine biological stations that use unfiltered or partially filtered sea water. The collecting technique is described below, but for practical reasons, it would be more convenient to obtain a subculture from some other laboratory; cultures are maintained by the Department of Biology, California State University, Long Beach, Long Beach, California, and by the U.S. Fish and Wildlife Service, Corpus Christi, Texas. *Dinophilus gyrociliatus* has been used in toxicological studies with metals (**31**), pesticides (**32**), and sediment pore water (**33**).

X4.2 *Collecting Techniques*—The collecting techniques used for *Ophryotrocha diadema* are identical to those used for *Dinophilus gyrociliatus*, and frequently they can be collected at the same time. Fouling communities attached to floating boat docks or pilings in protected waters are collected and placed in a bucket containing seawater. The material should be swished around vigorously in the bucket; during this procedure, large animals, such as mussels and tunicates, should be separated from one another, washed, and discarded. The remaining material in the bucket is passed through a 5.0-mm sieve. The material retained on the sieve is discarded. Approximately 500 to 700 mL of the material that passed through the sieve is placed in a 2-L graduated cylinder. The cylinder is filled to near the top with clean seawater. The vessel is sealed with an air-tight stopper and placed in a dark place at room temperature for 4 to 8 h or overnight. As the dissolved oxygen is depleted in the cylinder, *Dinophilus gyrociliatus*, as well as other animals, migrate to the top of the cylinder. Pipette the material from the top of the cylinder and examine under a dissecting microscope. *Dinophilus gyrociliatus* (Fig. X4.1) are white in appearance and lack jaws (which readily distinguishes it from *Ophryotrocha* spp.). Pipette the specimens into a second petri dish containing filtered seawater (through a 0.45-µm filter). Since specimens may be injured during this process, do not place more than five specimens in a petri dish. Examine the dishes each day, and remove any dead specimens; do not feed until 24 to 48 h after collection.

X4.3 *Feeding Requirements*—Frozen spinach, fish food flakes, and different green algal species have been used as food for *Dinophilus*. They are prepared in the same manner. The food is placed in a blender and chopped finely. When completed, the material is allowed to settle in the blender for approximately 5 min, at which time the supernatant fluid is poured into a container and used as food. A few drops of the supernatant are pipetted into each dish containing animals; the amount of food given will depend on the size of the population. If the population is maintained at room temperature, they need to be fed only once per week.

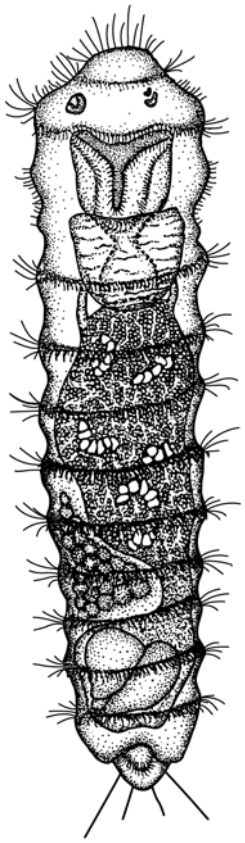

FIG. X4.1 *Dinophilus gyrociliatus*, Dorsal View of Adult Worm

X4.4 *Culture Techniques for Dinophilus gyrociliatus*—The life cycle of this species is short, requiring only 10 days for a complete cycle from egg to egg. Sexual dimorphism is conspicuous in *Dinophilus*. Two to five eggs are deposited in a gelatinous egg capsule in which there is usually one small egg and the remaining ones large (Fig. X4.2). The small egg develops into a dwarf male, and the large ones develop into females. The males and females break free from their individual egg membrane, and, before they emerge from the capsule, the male copulates with the females within the capsule. The male dies and the females emerge from the capsule after completion of copulation. Stock colonies have been cultured in 1 and 3.78-L jars. Cultures should be reestablished every 4 to 8 weeks to prevent overcrowding and to maintain healthy cultures. Cultures should be fed 5.0 mL of food every 1 to 2 weeks.

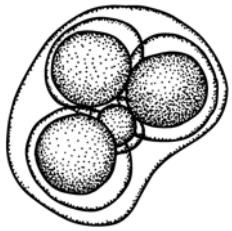

FIG. X4.2 *Dinophilus gyrociliatus*, Developing Embryos in a Mucoid Capsule; Three Large Embryos Will Develop Into Females and the One Small Embryo Will Develop Into a Male

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