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Standard Guide for Conducting Static and Flow-Through Acute Toxicity Tests With Mysids From the West Coast of the United States[1]

This standard is issued under the fixed designation E 1463; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining data concerning the adverse effects of a test material (not food) added to marine and estuarine waters on certain species of marine and estuarine mysids during 96 h of continuous exposure. Juvenile mysids used in these tests are taken from cultures shortly after release from the brood and exposed to varying concentrations of a toxicant in static or flow-through conditions. These procedures will be useful for conducting toxicity tests with other species of mysids, 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, results of tests conducted using unusual procedures are not likely to be comparable to results 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 tests with other species of mysids.

1.3 The procedures given in this guide 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 acute 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.

1.4 Results of acute toxicity tests with toxicants experimentally added to salt and estuarine waters should usually be reported in terms of a LC50 (median lethal concentration).

1.5 This guide is arranged as follows:

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1.6 The values stated in SI units are to be regarded as the standard.

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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]

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[3]

E 729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E 943 Terminology Relating to Biological Effects and Environmental Fate

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E 1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E 1203 Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology

3. Terminology

3.1 The terms "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 only used in connection with factors that directly relate to the acceptability of the test (see Section 13).

3.1.1 "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.

3.1.2 "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions:*

3.2.1 *LC50*—the statistically or graphically derived best estimate of the concentration of a toxicant added to an aqueous solution that results in the death of 50 % of the test organisms within the test period (see Definition E 943).

3.2.2 For definitions of other terms used in this guide, refer to Guide E 729, Terminology E 943, Guides E 1191 and E 1192, and Practice E 1203. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 The toxicity of a substance in marine or estuarine waters can be determined through a 96-h acute static test or a flow-through test. Static tests may be conducted in glass 2-L finger bowls or 350-mL finger bowls. Either size finger bowl provides a large surface-to-volume ratio and ample horizontal space to minimize cannibalism. The dishes should be covered and aerated. Whichever static technique is used, specific data on the concentration of test material are obtained and analyzed to determine the effect(s) of the toxicant on survival. In the flow-through acute technique, the test solution flows through the test chamber on a once-through basis throughout the test. The flow-through test is considered to be more representative of actual field conditions, but not all laboratories have the capabilities of conducting this type of test.

5. Significance and Use

5.1 Mysids are an important component of both the pelagic and epibenthic community. They are preyed upon by many species of fish, birds, and larger invertebrate species, and they are predators of smaller crustaceans and larval stages of invertebrates. In some cases, they feed upon algae. Mysids are sensitive to both organic and inorganic toxicants **(1)**.[4] The ecological importance of mysids, their wide geographical distribution, ability to be cultured in the laboratory, and sensitivity to contaminants make them appropriate acute toxicity test organisms.

5.2 An acute toxicity test is conducted to obtain information concerning the immediate effects of a short-term exposure to a test material on a test organism under specified experimental conditions. An acute toxicity test provides data on the short-term effects that are useful for comparisons to other species but does not provide information on delayed effects.

5.3 Results of acute toxicity tests can be used to predict acute effects likely to occur on aquatic organisms in field conditions except that mysids might avoid exposure when possible.

5.4 Results of acute toxicity tests might be used to compare the acute sensitivities of different species and the acute toxicities of different test materials, and to study the effects of various environmental factors on results of such tests.

5.5 Results of acute toxicity tests might be an important consideration when assessing the hazards of materials to aquatic organisms (see Guide E 1023) or when deriving water quality criteria for aquatic organisms **(2)**.

5.6 Results of acute toxicity tests might be useful for studying biological availability of, and structure activity relationships between test materials.

5.7 Results of acute toxicity tests will depend, in part, on the temperature, quality of the food, condition of test organisms, test procedures, and other factors.

[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] Withdrawn.

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

6. Apparatus

6.1 *Facilities*—Aquaria or tanks containing either clean (uncontaminated) natural sea water or reconstituted sea water (see 8.2) should be used for holding mysids after field collection and prior to a test. Both static-recirculating and flow-through holding systems have been used successfully **(1)**. Cultures of *Holmesimysis costata* have not been reported for media of reconstituted sea water. The holding tanks and any area used for manipulating live mysids should be located in a room or space separated from that in which toxicity tests are to be conducted. The sea water should be monitored periodically to ensure a constant salinity. The holding tanks, water supply, or the room in which they are kept should be equipped with temperature control. Aeration should be provided to ensure that the concentration of dissolved oxygen is greater than 60 % of saturation as well as adequate water circulation in the tanks. A timing device should be used to provide a 16-h light and an 8-h dark photoperiod. A 15 to 30-min transition period **(3)** when lights go on might be desirable to reduce the possibility of organisms being stressed by large, sudden increases in light intensity. A transition period when the lights go off might also be desirable.

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 adversely affect test organisms. In addition, equipment and facilities that contact stock or test solutions should be chosen to minimize sorption of test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastics should be used whenever possible to minimize dissolution, 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 a week or more before use **(4)**. 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 needed to remove rust particles. A specially designed system is usually necessary to obtain salt water from a natural water source **(5)**. Brass, copper, lead, galvanized metal, and natural rubber should not contact 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 adversely affect either survival, growth, or reproduction of mysids.

6.3 *Metering System*:

6.3.1 For flow-through tests, the metering system should be designed to accommodate the type and concentration(s) of the test material and the necessary flow rates of test solutions. The system should permit the mixing of test material with dilution water immediately before entrance to the test chambers and permit the supply of the selected concentration(s) of test material (see 9.1.3 and 11.7.5) in a reproducible fashion. Various metering systems, using different combinations of syringes, "dipping birds," siphons, pumps, saturators, solenoids, valves, and so forth, have been used successfully to control the concentrations of test material in, and the flow rates of, test solutions **(6)**. Proportional diluters **(7)** use an intermittent flow design and various devices for metering the test material **(8)**. Continuous-flow metering systems are also available, as are systems that prepare the different test solutions independently of each other **(6)**.

6.3.2 The metering system should be calibrated before and after the test by determining the flow rate through each test chamber and by measuring either the concentration of test material in each test chamber or the volume of solution used in each portion of the metering system. The general operation of the metering system should be visually checked daily in the morning and afternoon throughout the test. The metering system should be adjusted during the test if necessary.

6.3.3 The flow rate through each test chamber should be at least five-volume additions per 24 h. It is usually desirable to construct the metering system so that it can provide at least ten-volume additions per 24 h in case the loading is high (see 11.4) or there is rapid loss of test material due to microbial degradation, hydrolysis, oxidation, photolysis, reductions, sorption, or volatilization. At any particular time during the test, the flow rates through any two test chambers should not differ by more than 10 %.

6.4 *Test Chambers*:

6.4.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. Screens and cups may be used to create two or more compartments within each chamber but such compartments are not replicate experimental units. Therefore, the test solution can be in contact with the test organisms in the compartments within a test chamber, but, by definition, cannot flow from one chamber to another. Because the solution can flow from one compartment to another in the same test chamber, the temperature, concentration of test material, or types of pathogens and extraneous contaminants are likely to be more similar between compartments in the same test chamber than between compartments in different test chambers in the same treatment. Chambers should be covered to keep out extraneous contaminants and to reduce evaporation of test solution and test material. All chambers and compartments in a test must be identical.

6.4.2 At least two test chambers (in which compartments may be placed) should be used for each concentration and can consist of standard 57-L aquaria or can be constructed by gluing strong window glass with clear silicone adhesive. Because adhesives can sorb some organochlorine or organophosphorus pesticides, as little adhesive as possible should be used. If extra beads of adhesive are needed for strength, they should be placed on the outside of the chamber rather than on the inside.

6.4.3 Two methods have been used for conducting static acute toxicity tests with mysids **(1)**. The methods have used finger bowls as the test chamber, one measuring 2 L, the other 350 mL. Twenty animals are placed within the 2-L bowl, and ten animals in the 350-mL bowl; each bowl constitutes a replicate.

6.4.4 A flow-through test chamber can be a 2.5-L wide-mouth glass jar with a central stand-pipe. The test solution enters the compartment directly and flows through the stand-pipe into a drain. The stand-pipe should be covered with a 200 to 235-µm mesh nytex or nylon screen to avoid escapage of the young mysids.

6.5 *Cleaning*—The metering system, test chambers, and other glassware, and equipment used to store and prepare dilution water, stock solutions, and test sediments should be cleaned before use. New items should be cleaned before each use by washing with laboratory detergent, rinsing with water, a water-miscible organic solvent, water, and acid (10 % hydrochloric acid), and rinsed twice with distilled water, deionized, or dilution water. 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 be immediately emptied, rinsed with water, cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and solvents to remove organics), and rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits, and 200 mg of hypochloride (ClO') per litre is often used to remove organic matter and for disinfection. (A solution containing about 200 mg of ClO' may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water. However, ClO' is quite toxic to many aquatic animals **(9)** and is difficult to remove from some construction materials. It is often removed by soaking in a sodium thiosulfate, sodium sulfite, or sodium bisulfite solution, by autoclaving in distilled water for 20 min, or by drying the item and letting it sit for at least 24 h before use. An item cleaned or disinfected with hypochlorite should not be used unless it has been demonstrated at least once that unfed individuals of a sensitive aquatic species do not show more signs of stress, such as discoloration, unusual behavior, or death, when held for at least 48 h in static dilution water in which the item is soaking than when held in static dilution water containing a similar item that was not treated with ClO'. The metering system and test chambers should be rinsed with dilution water just before use. Glassware used only for live animals, not exposed to toxicants, may be cleaned using only clean distilled or dilution water, since the use of detergents is sometimes detrimental to live organisms.

6.6 *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 facilities, water, control, and handling techniques are adequate to result in acceptable (90 %) control level survival 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 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 safety glasses. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans **(10)**, recommended handling procedures **(11)**, and chemical and physical properties of test material should be studied before a test is begun. Special precautions might be necessary with radiolabeled test materials **(12)** and with test materials that are, or are suspected of being, carcinogenic.

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

7.3 Although 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. Removal or degradations of test material might be desirable before 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 should be available in adequate supply, be acceptable to test organisms, be uniform in quality, and not unnecessarily affect results of the test.

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 in that the range of temperature and salinity encountered during the holding period do not adversely affect the survival of the test organisms. A better criterion for an acceptable dilution water is that in which the test species will survive, grow, and reproduce satisfactorily in it.

8.1.2 The quality of the dilution water should be uniform so that the test organisms are cultured or acclimated, and the test conducted in water of the same quality. In fresh water, the range of hardness should be less than 5 mg/L or 10 % of the average, whichever is higher. In salt water, the range of salinity should be less than 2 g/kg or 20 % of the average, whichever is higher.

8.1.3 The dilution water should not unnecessarily affect results of an acute test because of such things as sorption or complexation of test material. Therefore, except as in accordance with 8.1.4, the concentration of both total organic carbon (TOC) and particulate matter should be less than 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 a water that is naturally or artificially high in TOC or particulate matter or low in dissolved oxygen. If such a water is used, it is

important that adequate analyses be performed to characterize the water and that a comparable test be available to be conducted in a more usual dilution water to facilitate interpretation of the results in the special water.

8.2 *Source*:

8.2.1 *Reconstituted Water*—If a reconstituted water is used for tests, the reconstituted waters described in Table 1 and Table 2 should be used whenever possible. *Neomysis mercedis* cultures have not been reported for media of reconstituted fresh waters. If desired, salinity can be adjusted with a sea salt or reconstituted sea water (see Table 3). A salinity of 1 to 3 g/kg and pH = 7.7 is needed for cultures with *Neomysis mercedis. Holmesimysis costata* cultures have not been reported for media of reconstituted sea water. Other salinities may be used for studying the effects of water quality on results of toxicity test. Reconstituted water is prepared by adding a sea salt or specified amounts of reagent-grade chemicals **(13)** to high-quality water with conductivity less than 1 μΩ/cm and TOC less than 5 mg/L. Acceptable water can usually be prepared using properly operated deionizations, distillation, or reverse osmosis units. Conductivity should be measured on each batch and TOC or COD should be measured at least twice a year and whenever substantial changes might be expected. If the water is prepared from a surface water source, TOC or COD should be measured on each batch. The reconstituted water should be intensively aerated before use. Problems have been encountered with some species of fish and invertebrates in some fresh and salt reconstituted waters, but sometimes these problems have been overcome by aging the reconstituted water for one or more weeks.

8.2.2 *Natural Dilution Water*:

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

8.2.2.2 The hardness, salinity, pH, and so forth. of a water may be adjusted, if desired, by addition of appropriate reagent-grade chemicals, sea salt, acid, base, distilled or deionized water, and so forth. When necessary, sea salt may be added to salt water to adjust salinity, 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 in the preparation of water for toxicity tests because residual chlorine and chlorine-produced oxidants are highly toxic to many aquatic animals **(9)**. Dechlorinated water should not be used 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 **(14)**. Some organic chloramines, however, react slowly with sodium bisulfite **(15)**. In addition to

TABLE 2 Quantities of Reagent-Grade[5] Chemicals to Be Added to Aerated Soft Reconstituted Fresh Water to Buffer pH (From Guide E 729)

NOTE 1—The solutions should not be aerated after addition of these chemicals.

pH[A]	Millilitres of Solution to Add to 15 L of Soft Water		
	1.0 *N* NaOH Solution	1.0 *M* KH_2PO_4 Solution[B]	0.5 *M* H_3BO_3 Solution[B]
6.0	1.3	80.0	...
6.5	5.0	30.0	...
7.0	19.0	30.0	...
7.5	...	...	...
8.0	19.0	20.0	...
8.5	6.5	...	40.0
9.0	8.8	...	30.0
9.5	11.0	...	20.0
10.0	16.0	...	18.0

[A]Approximate equilibrium pH with fish in water.

[B]Buffers containing ions such as phosphate and borate should not be used when conducting tests on metals unless it has been shown that the buffers do not affect the toxicity of the metals to the test species.

TABLE 3 Reconstituted Salt Water

NOTE 1—Add the following reagent-grade[5] chemicals in the amounts and order listed to 890 mL of water. Each chemical must be dissolved before the next is added[A] (from Guide E 729).

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
Na_4EDTA[B]	1 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 should be 8 ± 0.2. The desired test salinity is attained by dilution at time of use.

[B]Tetrasodium ethylenediaminetetraacetate. This should be omitted when toxicity tests are conducted on metals. When tests are conducted with fish or bivalve mollusc larvae, zooplankton, or crustaceans, the EDTA should be omitted, and the reconstituted salt water stripped of trace metals **(15)**.

TABLE 1 Quantities of Reagent-Grade[5] Chemicals Required to Prepare Reconstituted Fresh Waters and the Resulting Water Qualities (From Guide E 729)

Name	Salts Required, mg/L				pH[A]	pH[B]	Hardness[C]	Alkalinity[C]
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl				
Very soft	12	7.5	7.5	0.5	6.7–6.8	6.4–6.9	10–13	10–13
Soft	48	30.0	30.0	2.0	7.3–7.5	7.2–7.6	40–48	30–35
Hard	192	120.0	120.0	8.0	7.8–8.0	7.6–8.0	160–180	110–120
Very hard	384	240.0	240.0	16.0	8.0–8.2	8.0–8.4	280–320	225–245

[A]Approximate equilibrium pH after aeration.

[B]Approximate equilibrium pH after aeration and with fish in water.

[C]Expressed as mg $CaCo_3$/L.

residual chlorine, municipal drinking water often contains unacceptably high concentrations of metals, and quality is highly variable. Excessive concentrations of most metals can usually be removed with chelating resin **(16)**, but use of a different dilution water might be preferable. If dechlorinated water is used as dilution water or in its preparation, during the test either it must be shown that a sensitive aquatic species will survive, grow, and reproduce acceptably in it, or it must be shown at least three times each week on nonconsecutive days that fresh samples of dilution water either mysids (less than seven days postrelease from brood sac) do not show more signs of stress, such as discoloration, unusual behavior, or death, when held in the water for at least 48 h without food than when similarly held in a water that was not chlorinated and dechlorinated, or the concentration of residual chlorine in fresh water is less than 11 µg/L or the concentration of chlorine-produced oxidants in salt water is less than 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 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 % of saturation **(17)** to help ensure that dissolved oxygen concentrations in the test chambers are acceptable. Supersaturation by dissolved gases, that might be caused by heating the dilution water, should be avoided to prevent gas bubble disease **(18)**.

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 mysid predators.

8.4 *Characterization*—The following items should be measured at least twice each year or more often if such measurements have not been made semi-annually for at least two years or if surface water is used.

8.4.1 *All Waters*—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.

8.4.1.1 For the purposes of 8.4.1, the term "organophosphorus pesticides" refers to but is not limited to chloropyrifos, demetron, 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, dicambra, 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 PCBs and the organochlorine pesticides as listed, a chlorine detector is used instead of an electron capture detector to measure compounds that elute just before lindane to just after mirex. Organic chlorine does not refer only to chlorine associated with organochlorine pesticides and PCBs; it refers to all chlorine that elutes within the specified period.

8.4.2 *Estuarine Water*—Hardness, alkalinity, conductivity, sodium, and chloride.

8.4.3 *Salt Water*—Salinity or chlorinity.

8.4.4 The methods used should either be accurate and precise enough to adequately characterize the dilution water or have detection limits below concentrations that have been shown to adversely affect estuarine and marine mysids **(19)**.

9. Test Material

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

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 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 concentration(s) of the test material,

9.1.4 Estimate of toxicity to humans,

9.1.5 Recommended handling procedures (see section 6.11), and

9.1.6 Estimate of acute toxicity to test species.

9.2 *Stock Solution*:

9.2.1 In some cases the test solution can be added directly to the dilution water, but usually it is dissolved in a solvent to form a stock solution that is then added to dilution water. If a stock solution is used, the concentration and stability of the test material in it and 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. If the salinity of the dilution water will not be affected, deionized or distilled water may be used. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(20)**. 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. Use of more soluble form of the test material, such as a chloride or sulfate salts 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 mysids. Triethylene glycol is often a good organic solvent

[5] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, D.C. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory U.K. Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

for preparing stock solutions because of its low toxicity to aquatic animals **(21)**, low volatility, and high ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms, and acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[5] or better and its concentration in any test solution should not exceed 0.5 mL/L. 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 the preparation of the stock solution.)

9.2.4 If no solvent other than water is used then a dilution water control must be included in the test and 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 less.

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, 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 a dilution water control should 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 less in the solvent control and should be 10 % or less 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 less 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 possible effects of solvent on the results of the test.

9.3 *Test Concentration(s)*:

9.3.1 If the test is intended to allow calculation of an LC50, the test concentrations (see 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 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 that are 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 at the solubility limit; such information is certainly worth knowing.

9.3.2 In some (usually regulatory) situations, it is only necessary to determine whether a specific concentration of test material is acutely toxic to the test species, or whether the LC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in surface water, the concentration resulting from the 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 only to test that concentration (see 11.1.1.2), 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. Both *Holmesimysis costata* and *Neomysis mercedis* have been successfully maintained and cultured in the laboratory and tested using the following procedures **(1)**. These species should be identified using the information summarized in Appendix X1 and Appendix X2.

10.2 *Age*—Mysids used in acute toxicity tests should be three to seven and one to five days postrelease from the brood sac for *Holmesimysis costata* and *Neomysis mercedis*, respectively.

10.3 *Source*—All mysids used in a test must be from the same brood stock, either hatched and raised in the laboratory or brought into the laboratory. The mysid neonates used in a test must have been released in the laboratory. These species should be collected and handled using the information summarized in Appendix X1 and Appendix X2. Neonates from the first type of brood stock are preferable because the mysids are acclimated to laboratory conditions for one or more generations and the acceptability of the food, water, and handling procedures before the test is begun will be demonstrated.

10.4 *Brood Stock*:

10.4.1 Brood stock may be obtained from another laboratory or a wild population from an unpolluted area. When brood stock is brought into the laboratory, it should be placed in a tank along with the water in which it was transported. The temperature should then be changed at a rate not to exceed 3°C within 12 h (preferably not more that 3°C within 72 h) and the salinity should be changed at a rate not to exceed 3 g/kg within 12 h.

10.4.2 West coast species of mysids have been cultured in filtered sea water **(1)**. *Holmesimysis costata* cultures have been maintained for several generations under static conditions in 113.5-L (30-gal) aquaria containing natural, filtered sea water. Each aquarium is provided with under-gravel filters, a layer of

substrate (medium grade oyster shell) 2 cm in thickness, and gentle aeration at each corner that provides adequate dissolved oxygen and a current conducive to feeding. Several "mysid generators" have been used successfully for obtaining neonates (see Guide E 1191). One design uses hard plastic egg crate with openings of 1.4 cm covered with a 250-µm nylon mesh cloth and placed on top of the substrate. A 4-L egg crate box with legs, covered with a 500-µm nylon mesh cloth, is placed within the aquarium. Brood stock is placed within this box that allows the young to fall through the mesh covering and live in the main body of the aquarium. *Neomysis mercedis* cultures have been maintained for several generations in circular tanks under flow-through conditions. Gravid females of both species of mysids are removed and isolated when neonates are needed.

10.4.3 To maintain mysids in good condition and avoid unnecessary stress, brood stock should not be subjected to rapid changes in temperature, photoperiod, or water quality. Mysids should not be subjected to more than a 3°C change in temperature or a 3 g/kg change in salinity in any 12-h period. The concentration of dissolved oxygen should always be above 60 % of saturation.

10.4.4 Reproduction will be depressed when the culture density is high. This phenomenon has not occurred when maintained at densities of 10 mysids/L or less. Therefore, when cultures are not being used for supplying test organisms, enough adults should be removed at least every two weeks to stimulate reproduction. It is desirable to keep neonates, juveniles, and adults of both species of mysids in separate tanks.

10.4.5 Brood stock tanks should be kept free of other animals, such as hydroids and worms. If an outbreak of these animals or others occurs, all mysids should be removed and the tank thoroughly cleaned. The substrate should either be washed and dried, autoclaved or discarded. Salinity and temperature should be appropriate for the particular species and consistent with the specified test conditions (see 11.3 and 11.5).

10.5 *Food*—At least once daily, mysids in brood stock tanks and in test chambers should be fed live brine shrimp nauplii (see Practice E 1203) in excess, in order to maintain live nauplii in the chambers at all times to prevent cannibalism and support adequate survival, growth, and reproduction in the brood stock. The ration should be adjusted in accordance with the number of mysids in the stock colony. A ration of 150 nauplii per mysid per day has been used successfully **(1)**. A regime of 75 nauplii per mysid twice a day or 50 nauplii three times a day might improve growth and reproduction in the brood stock. It is desirable to add a piece of fresh, carefully washed frond of the giant kelp *Macrocystis* to the brood stock of *Holmesimysis costata* that provides additional substrate and food for the mysids. The kelp should be analyzed for any contaminant that might reasonably be expected to occur in the kelp bed. It is desirable to supplement the diet of *Neomysis mercedis* with commercially available food having micronutrients and vitamins[6]; supplement of rotifers or algae may also be desirable.

10.6 *Handling*—Mysids should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that the mysid is not necessarily stressed. Dip nets are best for removing gravid female mysids from the brood stock tanks. Such nets are commercially available; the mesh opening should not be greater than 250 µm. Mysids that touch dry surfaces, dropped, injured, or have a marine leech attached (an external parasite) should be discarded.

10.7 *Harvesting Young*—As young leave the marsupium of the female, they fall through the openings of the mysid generator (see Guide E 1191) into the main part of the aquarium or tank (see 10.4.2). Young mysids may then be picked up with a 5-mm bore pipette and transferred to a container for separation prior to beginning a test.

10.8 *Quality*—Mysids three to seven days (*Holmesimysis costata*) or one to five days (*Neomysis mercedis*) post-release are satisfactory for use in an acute toxicity test. Representative mysids from the brood stock should be analyzed for the test material, if it might be present in the environment.

11. Procedure

11.1 *Experimental Design*:

11.1.1 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).

11.1.1.1 An acute test intended to allow calculation of a LC50 usually consists 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, 9.2.4, 9.2.5) mysids 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 an LC50 for several durations (see 11.10.3) and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low.

11.1.1.2 Although most acute toxicity tests use five test concentrations plus control(s), in some instances it might be necessary only to determine whether a specific concentration affects survival. If this is the case, then only that concentration and the control(s) are necessary. Two additional concentrations

[6] The sole source of supply of the apparatus known to the committee at this time is TetraMarin, a product of TetraWerke Germany. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

11.1.2 The primary focus of the physical and experimental design of the test and the statistical analysis of the data is the experimental unit, that is defined as the smallest physical entity to which treatments can be independently assigned **(22)**. 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 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. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, that may be in a row or a rectangle) is preferable to a completely randomized design.

11.1.3 The effect of the test material on survival cannot be determined accurately if any factor that affects survival is too dissimilar between experimental units. Since three to seven days (*Holmesimysis costata*) and one to five days (*Neomysis mercedis*) postrelease neonates are used in the experiment, and since the sex of the mysid cannot be determined at this age, it is impossible to determine if there is any sexual difference in the effect of a particular toxicant.

11.1.4 The minimum desirable number of test chambers and organisms per treatment should be calculated from the expected variance within test chambers, the expected variance between test chambers within a treatment, and the maximum acceptable width of confidence interval on the LC50 **(22)**. 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 ten organisms should be exposed to each treatment in static and renewal tests, and at least 20 organisms in flow-through tests. Organisms in a treatment should be divided between two or more test chambers to allow estimation of experimental error **(23)**. If the controls are important in the calculation of results, such as correcting 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.

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

11.2 *Dissolved Oxygen*:

11.2.1 The concentration of dissolved oxygen in each test chamber must be from 60 to 100 % of saturation **(17)** during the entire test.

11.2.2 Test solutions may be gently aerated during static and renewal tests if the concentration of test material in the aerated test chamber at the end of the test is not more than 20 % lower than that in a comparable unaerated test chamber. Test solutions may be gently aerated during flow-through tests if the concentrations of test material are measured according to 11.11.1.2. Turbulence, however, should be avoided because it might stress test organisms, resuspend fecal matter, and greatly increase volatilization. Because aeration readily occurs at the surface, efficient aeration can be achieved with minimum turbulence by using an air lift to transfer solution from the bottom to the surface. 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 if aeration affects the result of the test.

11.3 *Temperature*:

11.3.1 Tests with *Holmesimysis costata* should be conducted at a temperature range from 13 to 19°C and *Neomysis mercedis* at a temperature range from 15 to 19°C. It may be necessary to conduct tests with *Holmesimysis costata* at a temperature resembling its geographical distribution (17 and 15 ± 2°C for south and north of point conception, respectively).

11.3.2 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.

11.4 *Loading*:

11.4.1 The grams of organisms (whole body, wet weight, 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 the concentration of dissolved oxygen and test material do not fall below acceptable levels, concentrations of metabolic products do not exceed acceptable levels, and the test organisms are not stressed because of aggression or crowding.

11.4.2 A lower number of test organisms should be used if aggression occurs.

11.5 *Salinity*—The salinity in the toxicity tests must be within the tolerance range of the selected species of mysid. The optimum salinity for *Holmesimysis costata* is 30 to 35 g/kg and 1 to 3 g/kg for *Neomysis mercedis* **(1)**. If a test salinity other than the optimum salinity is used, then an additional control at the optimum salinity must be employed.

11.6 *Light*—The light in the laboratory should be maintained at 16-h light-8-h dark photoperiod.

11.7 *Beginning the Test*:

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

11.7.2 A representative sample of the test organism must be either randomly distributed 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 assigned by random assignment of one organism

to each chamber, 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 add them to the test chamber all at once.

11.7.3 On the day that the toxicity test is initiated, a sufficient number of mysids should be removed from the holding facility at one time to provide about one third more animals than are needed. Select a set of test chambers (one test chamber from each test concentration plus control(s)) to be processed together to avoid possible selective bias during loading. The mysids should be transferred using a wide borer (larger than the largest mysid) glass pipette with a smooth tip. Mysids should be handled gently to avoid injuries.

11.7.4 Static tests should begin by placing test organisms in the chambers within 30 min after the test material was added to the dilution water. If the test material forms a film on the surface of the test solution, static and renewal tests may be begun by placing test material in the test chambers 18 to 24 h after the test organisms were added. In an alternative procedure, the dilution water with organisms in it may be gently aerated in the chambers, but aeration must be stopped before addition of test material except in accordance with 11.2.2.

11.7.5 Flow-through tests should be begun by either placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of test material to have reached steady state, or activating the metering device in the metering system several days after organisms were placed in test chambers that had dilution water flowing through them. This second alternative requires the addition of a "spike," that is, an aliquot of test material sufficient to establish the desired test concentration in the test chamber at the time of activation of the metering device. The first alternative allows the investigator to study the properties of the test material (see 11.11.2) and the operation of the metering system immediately prior to the test, whereas the second alternative allows the organisms to partially adjust to the chambers before the beginning of the test.

11.8 *Feeding*—In static tests mysids should be fed live brine shrimp nauplii to excess once each day (see 10.5). In flow-through tests mysids are fed brine shrimp nauplii three times a day at a rate of 30 nauplii per mysid. Dead nauplii should be removed daily from each chamber to prevent the build up of nitrogenous material.

11.9 *Duration of Test*—The 96-h duration of the test is timed from initiation of the experiment. At the end of the test it might be desirable to place the live test organisms in dilution water that does not contain any added test material for two to eight days and feed them to determine whether delayed effects occur.

11.10 *Biological Data*:

11.10.1 The criteria for death of mysids are opaque white coloration, immobility (especially absence of movement of respiratory and feeding appendages), and lack of reaction to gentle prodding. Dead mysids 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.

11.10.2 Live test organisms should not be stressed in an attempt to determine whether they are dead, immobilized, or otherwise affected. Prodding of organisms and movement of test chambers during test should be done very gently. Some organisms exposed to some organophosphorus compounds seem to be very sensitive to sudden changes in light intensity.

11.10.3 The number of dead organisms in each test chamber should be counted every 24 h after the beginning of the test. If the shape of the toxicity curve is to be defined, counts should be performed more often; a suggested schedule is to count the number of dead organisms in each chamber, 3, 6, 12, and 24 h after the beginning of the test and twice a day thereafter to the end of the test. If test solutions are opaque, it might be necessary to insert a partition into the test chamber at the observation periods to move the test organisms to one end where they can be seen. If such a procedure is necessary, great care should be taken not to stress or damage live organisms or to cross-contaminate treatments. In some cases, for example, under conditions of extreme turbidity, the only way to obtain accurate counts before the end of the test is to terminate separate replicate test chambers each time counts are desired, but such a procedure is usually not worth the effort.

11.10.4 If it can be done without stressing live organisms, dead organisms should be removed at least once every 24 h.

11.10.5 All mysids used should be destroyed at the end of the test.

11.11 *Other Measurements*:

11.11.1 *Water Quality*:

11.11.1.1 *Static Tests*—If 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. In both waters, measurements of ammonia, particulate matter, total dissolved gas, and TOC are desirable. The dissolved oxygen concentration must be measured at the beginning and end of the test and at least every 48 h in between in the control and the high, medium, and low test concentrations as long as live organisms are present. The pH should be measured at the beginning and end of the test in the control and the high, medium, and low concentrations of test material.

11.11.1.2 *Flow-Through Tests*—Certain measurements should be performed at least at the beginning of the test, if data are available to show that the quality of the dilution water is constant, and daily if such data are not available. In tests with *Neomysis mercedis*, hardness, alkalinity, conductivity, and pH of dilution water should be measured, and 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. In both waters, measurement of ammonia, particulate matter, total dissolved gas, and TOC is desirable. The dissolved oxygen concentration must be measured at the beginning and end of the test and at least every 48 h in between in the control and the high, medium, and low test concentrations as long as live organisms are present. The pH should be measured at the beginning and end of the test in the control and in the high, medium, and low concentrations of test material.

11.11.2 *Temperature*:

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

11.11.2.2 In static and renewal tests, either in at least one test chamber, temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily, or 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 or monitored 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.

11.11.2.3 In flow-through tests, in at least one chamber either temperature must be measured or monitored at least hourly or the maximum and minimum temperatures must be measured daily. In addition, near both beginning and end of the test, temperature must be measured concurrently in all test chambers.

11.11.3 *Test Material*:

11.11.3.1 If the test material is uniformly dispersed throughout the test chamber, water samples should be taken by pipette or siphon 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 appropriate-sized containers from which the test material can be extracted or analyzed directly. If the test material is not uniformly dispersed in the test chamber in static and renewal tests, the whole volume of the solution in the test chamber should be used as a sample or treated appropriately (for example, by adding acid, base, or surfactant and mixing thoroughly) to uniformly distribute the test material before a sample is taken. If the test material is not uniformly dispersed in the test chamber in flow-through tests, a large volume of the solution flowing into the test chambers should be collected and used as the sample or treated appropriately to uniformly distribute the test material in the sample before a subsample is taken.

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

11.11.3.3 In static and renewal tests, the concentration of test material should be measured, if possible, in at least the control and high, medium, and low concentrations of test material at the beginning of the test (see 14.1). Measurement of degradations products might be desirable.

11.11.4 *Flow-Through Tests*:

11.11.4.1 The concentration of test material in the test chambers should be measured as often as practical during the test. The concentration of test material should be measured in all chambers concurrently at least once during the test preferably near the beginning of the test, except for the control treatment, each test chamber (especially for those concentrations closest to the LC50) at least one additional time during the test on a schedule designed to give reasonable confidence in the concentration of the material in the test chambers, taking into account the flow rate and the number of independent metering devices, and at least one appropriate chamber whenever a malfunction is detected in any part of the metering system.

11.11.4.2 In each treatment the highest measured concentration obtained during the test divided by the lowest should be less than 1.5. The variability of the sampling and analytical procedures should be determined before the beginning of the test to determine how many samples should be taken and analyses performed at each sampling point to ensure that the limit of 1.5 is not violated just because of sampling or analytical variability.

11.11.4.3 If the measured concentration of test material in any chamber is more than 30 % higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the metering system, the cause should be identified. Measurement of the concentration of the material in the solution flowing into the test chamber will indicate whether the cause is in the metering system or in the test chamber. If the concentration in the test chamber is too high, the stock solution might not have been calibrated correctly. If the concentration is too low, additional possible causes are microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization. A faster flow rate might be desirable (see 6.3.3). If the test organisms are probably being exposed to substantial concentrations of one or more impurities or degradation or reaction products, measurement of the impurities or products is 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 **(23)** to minimize 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 sensitive enough, methods should be obtained from other reliable sources **(24)**. The concentration of nonionized ammonia may be calculated indirectly from pH, temperature, and the concentration of total ammonia **(25)**.

12.3 Methods used to analyze food (see 10.5) or test organisms (see 10.8) should be obtained from appropriate sources **(26)**.

12.4 The analytical method used to measure the concentration of toxicant in test chambers must be validated before beginning the test. The precision and bias 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 An acute toxicity test should usually be considered unacceptable if one or more of the following occurred, except for example, 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 only measured a minimal number of times, one deviation of more than 3°C might indicate that more deviations would have been found if temperature has been measured more often and:

13.1.1 All test chambers and compartments were not identical,

13.1.2 Treatments were not randomly assigned 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 Young mysids used in the test were not obtained from animals that had been released in the laboratory,

13.1.6 Individual test organisms were not impartially or randomly assigned to test chambers or compartments,

13.1.7 More than 10 % of the organisms in any required control treatment showed signs of disease or stress, such as discoloration, unusual behavior, or death, during test,

13.1.8 Dissolved oxygen and temperature were not measured as specified in 11.11,

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

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

13.1.11 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,

13.1.12 At any one time, the difference between the measured temperatures in any two test chambers was more than 2°C, and

13.1.13 At any one time, if the salinity deviates beyond the optimum salinity for the species (30 to 35 g/kg for *Holmesimysis costata* and 1 to 3 g/kg for *Neomysis mercedis* unless as allowed in 11.5 by inclusion of another control.

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 mysids exposed to it.

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

14. Interpretation of Results

14.1 The LC50 and its 95 % confidence limits should be calculated on the basis of the measured initial concentrations of the test material if available, or the calculated initial concentrations for static tests, and the average measured concentrations of test material, if available, or the calculated average concentrations for renewal and flow-through tests.

14.2 Most acute toxicity tests produce quantal data; that is, counts of the number of alive or dead. A variety of methods **(27)** 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 0 and 100, but the most widely used are the probit, moving average, Spearman-Karber and Litchfield-Wilcoxon **(27)** methods. The method used should appropriately take into account the number of test chambers per treatment and the number of test mysids per chamber. The binomial test can usually be used to obtain statistically reliable information about the LC50 even when less than two concentrations kill between 0 and 100 %. The binomial method does not provide a point estimate of the LC50, but it does provide arrange within which the LC50 should lie. If desired, an interpolation procedure may be used to obtain an approximate LC50.

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

14.4 An LC near an extreme of toxicity, such as in 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 to report the highest concentration of test material that actually killed no greater a percentage of the test organisms than did the control treatment(s) or 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 percentage of the killed 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 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 tests of heterogeneity. Then a pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test.

15. Report

15.1 The record of the results of an acceptable acute toxicity test should include the following information either directly or by reference to other available documents:

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 obtained 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 and means and ranges of weights and lengths of the mysids at the beginning of the test,

15.1.5 Description of the experimental design, test chambers and covers, the depth and volume of the solution in the chambers, temperature, salinity, lighting, the method of beginning the test, and the number of mysids and chambers used per treatment. If a flow-through system is used, a description of the metering system and flow rate as volume additions per 24 h,

15.1.6 The 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 The averages and ranges of the acclimation and test temperatures and the method(s) of measuring or monitoring or both,

15.1.8 Schedule for obtaining samples of test solutions and the 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 LC50s and a summary of general observations on other effects,

15.1.11 A 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; the highest concentration of test material that killed or affected no greater a percentage of the test organisms than did the control treatment. Specify whether results are based on measured or unmeasured concentrations of the test material. For formulations and commercial products, specify whether results are based on whole mixture or active ingredient, and

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

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

16. Keywords

16.1 acute toxicity; aquatic; culture techniques; *Holmesimysis costata*; invertebrates; mysids; *Neomysis mercedis*

APPENDIXES

(Nonmandatory Information)

X1. HOLMESIMYSIS COSTATA

X1.1 *Ecological Requirements—Holmesimysis costata* (= *Acanthomysis sculpta*) is one of five species of the genus Holmesimysis that is present in the North Pacific Ocean. *Holmesimysis costata* is the principal species of the genus in California marine waters. This species can be separated from the other species of the genus by the structure of the dorsal surface of the abdominal region and by the distribution of setae on the telson (see Figs. X1.1-X1.15). *H. costata* has proved to be a useful test species for environmental studies **(1, 28, 29)**. The toxicity test should be conducted at 13 to 19°C using sea water with a salinity between 30 and 35 g/kg. (Tests should be conducted at 15 ± 2°C for organisms collected north of Point Conception and 17 ± 2°C for those collected south of Point Conception.) *Holmesimysis costata* lives in offshore kelp beds and can be easily collected.

X1.2 *Collecting and Handling Techniques*—This species occurs abundantly offshore among the fronds of the giant kelp especially during the summer months. Collections are made from a boat, and the mysids are captured by passing a hand net (0.5 to 1.0 mm mesh) through the kelp canopy. Specimens should be transferred to a 5-gal (3.79 L) bucket filled with sea water and then transported to the laboratory. In the laboratory, the contents of the bucket should be poured into one or more pans and *H. costata* are separated from the other organisms. Any specimen that is injured or does not appear to be in good condition should be eliminated. Some specimens might be parasitized externally by a marine leech; these specimens should not be used or placed in the laboratory stock colony. Mysids can be picked up using a bulb pipette with a 5-mm diameter.

X1.2.1 For acclimation, *H. costata* can be placed in an aquarium provided with aeration at a density of approximately 10 to 20 specimens/L of seawater. The water should be changed if it becomes cloudy.

X1.3 *Toxicity Test Specifications*—For obtaining young mysids, the adults should be placed in a cage within an

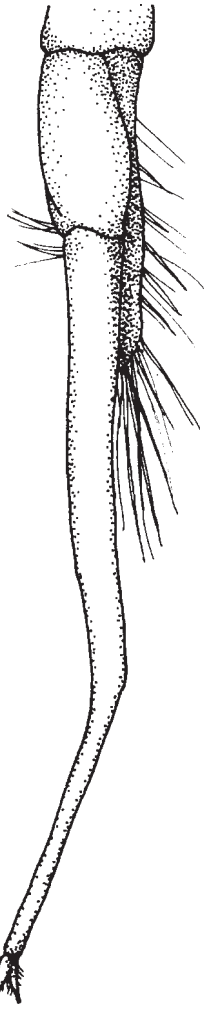

FIG. X1.1 Fourth Pleopod of Male *Holmesimysis costata* With a Close-Up View of the Terminal Segment

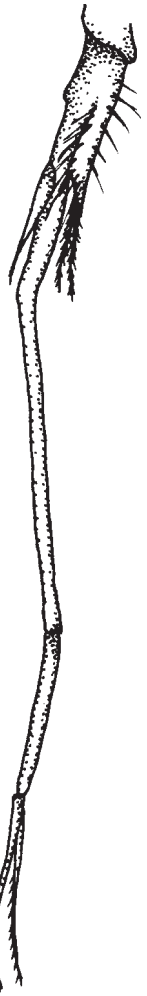

FIG. X1.2 Fourth Pleopod of a Member of the Genus *Acanthomysis* With a Close-Up View of Two Long Setae on the Terminal Segment

aquarium. The cage should be covered with nytex screening with a 0.25-mm mesh that allows the newborn to escape into the main body of the aquarium but retains the adults. The newborn can be removed from the aquarium with a fine dip net or glass pipette and transferred to a dish where specimens can be observed and removed for testing.

X1.4 *Life Cycle and Age Class—Holmesimysis costata* has a short life cycle and is capable of completing three or four life cycles a year under laboratory conditions. Females will produce more than one brood set under laboratory conditions. Animals can be cultured in the laboratory on a diet of *Artemia* nauplii larvae, powdered fish flake food, and fresh fronds of the giant kelp (*Macrocystis*).

X1.5 *Identification*:

X1.5.1 *Systematics of Holmesimysis*—Confusion has existed concerning in which genus to place the species of mysid used in marine toxicity tests in California. Up to 1988 all authors have referred to their test mysid species as *Acanthomysis sculpta* **(30)**. Holmquist **(31)** established the genus *Holmesimysis* and placed all known species of the genus *Acanthomysis* from the Pacific Coast of North America in the new genus. Kathman et al. **(32)** stated that the genus *Acanthomysis* does not occur in the Pacific Ocean. Consequently, references to *Acanthomysis sculpta* from the Pacific Coast must be treated with reserve. It may be *H. costata* or other members of this genus belong to some other genus. The genera *Holmesimysis* and *Acanthomysis* can be separated as follows:

X1.5.1.1 *Holmesimysis*—Exopod of Male Pleopod IV consists of two segments and terminates distally with two short spiny, peg-like structures (see Fig. X1.1). Known from the Pacific Ocean.

X1.5.1.2 *Acanthomysis*—Exopod of Male Pleopod IV consists of two segments and terminates distally in two long setae (see Fig. X1.2). Unknown from the Pacific Ocean.

X1.6 The five known species of *Holmesimysis* from the Pacific Coast of North America are separated as follows:

X1.6.1 *Holmesimysis costata* **(33)**—*Holmesimysis costata* **(33, 34, 35)**; *Mysis costata* **(33)**; *Acanthomysis sculpta* **(36)** and recent citations especially in marine bioassay reports from California; not *Neomysis costata***(30)**; and not *Acanthomysis costata* **(37, 38)**.

X1.6.1.1 *Holmesimysis costata* is separated from the other known species of the genus by the following characteristics:

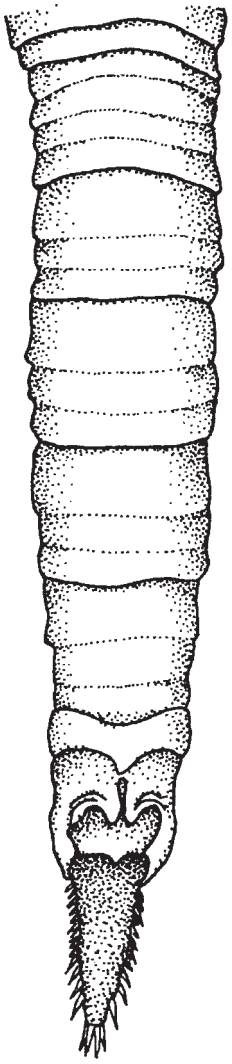

FIG. X1.3 *Holmesimysis costata*, Dorsal View, Abdominal Region With Telson

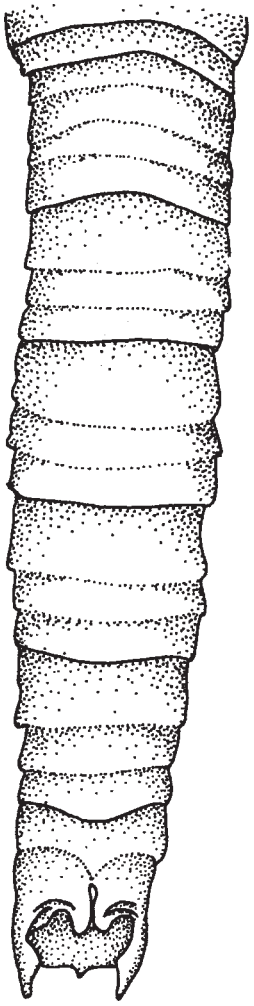

FIG. X1.4 *Holmesimysis costata*, Abdominal Region, Dorsal View

(a) *Abdominal Region (see Fig. X1.3 and Fig. X1.4)*—Segments 1 to 3 with two dorsal transverse folds and occasionally three folds; Segments 4 and 5 with two folds; Segment 6 with angular fold; posterior process on Segment 6.

(b) Telson (see Fig. X1.9)—Distal spines larger and thicker than the other known species of the genus.

X1.6.1.2 *Ecological Notes*—Found primarily in offshore giant kelp beds. It can occur with other species of the genus in the Pacific northwest; intertidal to subtidal.

X1.6.1.3 *Geographical Distribution*—British Columbia to Southern California.

X1.6.2 *Holmesimysis nuda (*39*)—Acanthomysis sculpta* var. *nuda***(39)**; and *Holmesimysis nuda* **(39, 34, 35, 39)**.

X1.6.2.1 *Holmesimysis nuda* is separated from the other species in the genus by the following characteristics:

(a) Abdominal Region (see Fig. X1.5)—Segments 1 and 2 without dorsal transverse folds; Segment 4 with one, sometimes two, dorsal folds; Segment 4 with zero, one, or two dorsal transverse folds; Segments 5 and 6 without dorsal folds; posterior processes on Segment 6.

(b) Telson (see Fig. X1.10)—Eighteen to 20 lateral spines per side but smaller than in *H. costata.*

X1.6.2.2 *Ecological Notes*—Collected from moderately exposed to protected beaches from a variety of substrates; can occur with *H. costata* and *H. nudensis*; found to a depth of a few metres.

X1.6.2.3 *Geographical Distribution*—British Columbia to Washington.

X1.6.3 *Holmesimysis nudensis (*31*)—Holmesimysis nudensis* **(31, 35)**.

X1.6.3.1 *Holmesimysis nudensis* is separated from the other species in the genus by these characteristics:

(a) Abdominal Region (see Fig. X1.6)—Segments 1 to 3 without dorsal folds; Segments 4 and 5 occasionally have dorsal transverse folds; Segment 6 usually lacks transverse folds; posterior process dorsally and occasionally laterally on Segment 5; posterior process on Segment 6.

(b) Telson (see Fig. X1.11)—Eighteen to 23 lateral spines per side and are smaller than those present in *H. costata* and *H. nuda*; the lateral, most posterior pair of larger spines extends beyond the apex of the telson.

X1.6.3.2 *Ecological Notes*—None. Occurred with *H. costata* and *H. nuda*.

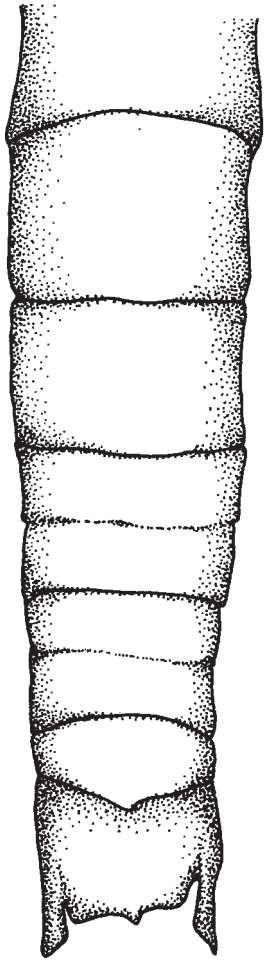

FIG. X1.5 *Holmesimysis nuda*, Abdominal Region, Dorsal View

X1.6.3.3 *Geographical Distribution*—Known from 100 specimens collected on two occasions from Graham Island, British Columbia.

X1.6.4 *Holmesimysis sculpta (*36*)—Holmesimysis sculpta* **(31, 35, 36)**; *Neomysis sculpta* (**(36)** in part); and *Acanthomysis sculpta* **(36, 37, 38, 39)**.

X1.6.4.1 *Holmesimysis sculpta* can be separated from the other species in the genus by these characteristics:

(a) Abdominal Region (see Fig. X1.7)—Segment 1 with three dorsal transverse folds; Segments 2 through 6 with two dorsal transverse folds; dorsal posterior processes on Segments 4 and 5; lateral posterior processes sometimes present on Segment 5.

(b) Telson (see Fig. X1.12)—About 18 larger spines per side but smaller than those in *H. costata* or *H. sculptoides*.

X1.6.4.2 *Ecological Notes*—None.

X1.6.4.3 *Geographical Distribution*—British Columbia and questionably from California.

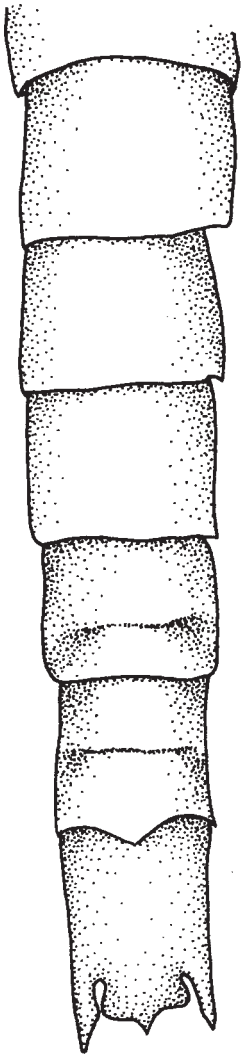

FIG. X1.6 *Holmesimysis nudensis*, Abdominal Region, Dorsal View

X1.6.5 *Holmesimysis sculptoides (*31*)—Holmesimysis sculptoides* **(31, 35)**; *Neomysis sculpta* (**(36)** in part), and *Acanthomysis sculpta* (**(36, 37, 38, 39)** in part).

X1.6.5.1 *Holmesimysis sculptoides* can be separated from the other species in the genus by these characteristics:

(a) Abdominal Region (see Fig. X1.8)—Segment 1 with two or three dorsal transverse folds; Segments 2 through 6 with two dorsal transverse folds; lateral processes on Segment 5; posterior process on Segment 6.

(b) Telson (see Fig. X1.13)—Eighteen to 20 larger spines per side with the most distal ones larger but thinner than in *H. costata*; spines larger in the other three species.

X1.6.5.2 *Ecological Notes*—None. Can occur with *H. costata*.

X1.6.5.3 *Geographical Distribution*—British Columbia and Washington.

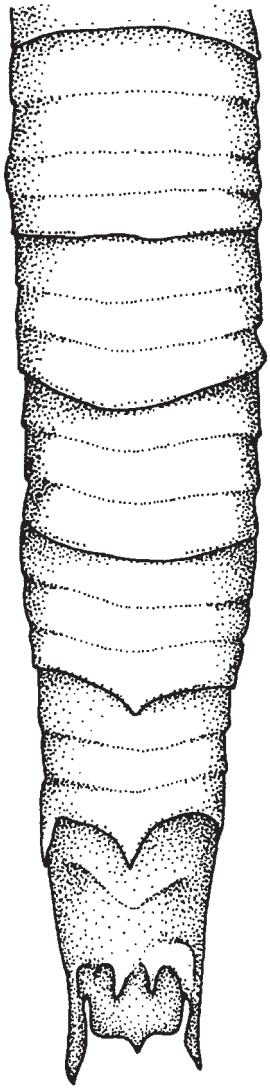

FIG. X1.7 *Holmesimysis sculpta*, Abdominal Region, Dorsal View

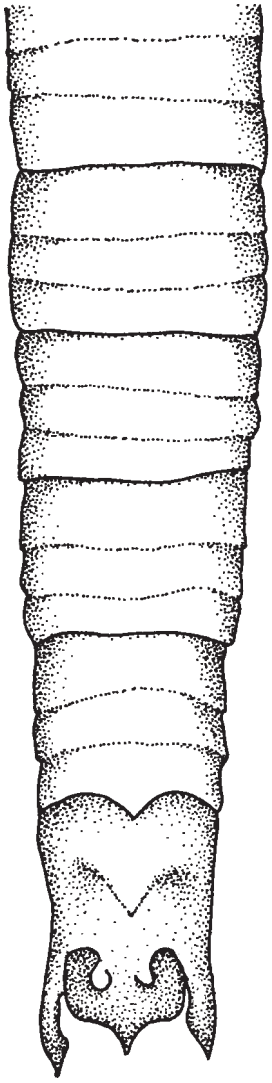

FIG. X1.8 *Holmesimysis sculptoides*, Abdominal Region, Dorsal View

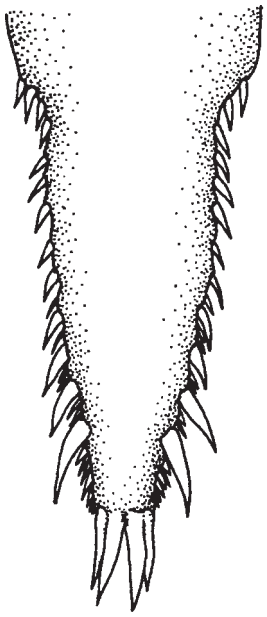

FIG. X1.9 *Holmesimysis costata*, Telson

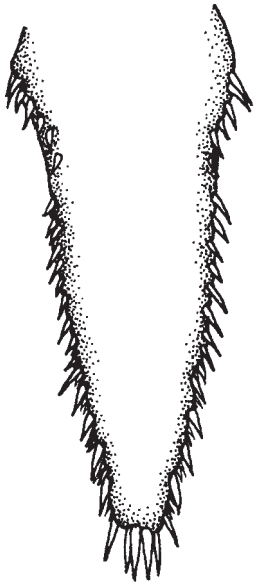

FIG. X1.10 *Holmesimysis nuda*, Telson

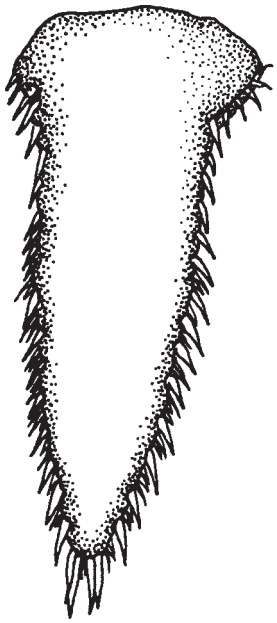

FIG. X1.11 *Holmesimysis nudensis*, Telson

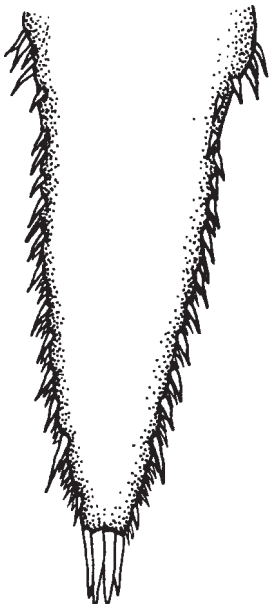

FIG. X1.12 *Holmesimysis sculpta*, Telson

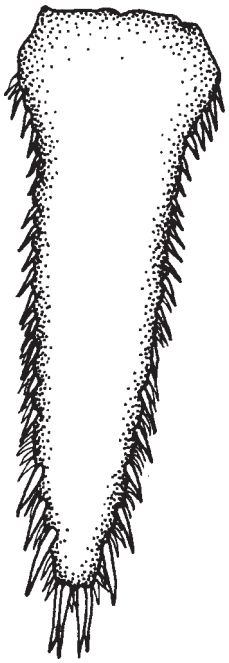

FIG. X1.13 *Holmesimysis sculptoides*, Telson

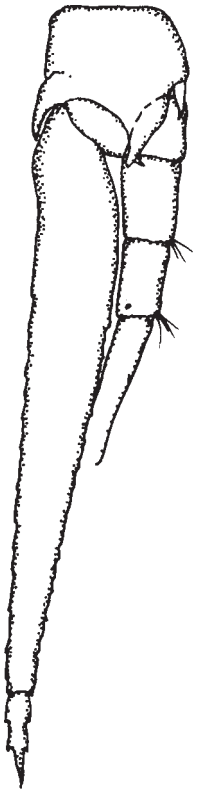

FIG. X1.14 *Neomysis mercedis*, Antennal Scale and Peduncle

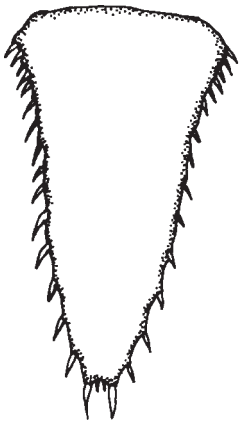

FIG. X1.15 *Neomysis mercedis*, Telson

X2. NEOMYSIS MERCEDIS

X2.1 *Ecological Requirements—Neomysis mercedis* ranges from Prince William Sound, Alaska, to south of Point Conception, California. Temperature and salinity ranges are from 6 to 22°C and fresh water to 18 g/kg, respectively **(40)**. Salinity appears to control distribution as they are most abundant between fresh water and 7.2 g/kg **(41)**. *N mercedis* has been used for toxicity tests with pesticides **(42, 43)**. The toxicity tests should be conducted at 17 ± 2°C using hard fresh water (150 to 200 mg/L $CaCO_3$ hardness and alkalinity) with added natural sea water or reconstituted salt water to 1 to 3 g/kg (2 g/kg preferred) **(1)**. *N. mercedis* lives in the Sacramento-San Joaquin Estuary and Lake Merced in the city of San Francisco **(44)**. It can be easily collected from both locations.

X2.2 *Collecting and Handling Techniques—N. mercedis* can be collected by hand dip nets or plankton tows in rivers and estuaries. Collection with a dip net (0.5 to 1.0-mm mesh) at night imports minimal mechanical damage. This method yields many specimens in good condition and with little accompanying debris. Specimens should be transferred to a 30-gal plastic can filled with site water and then transported to the laboratory. Aeration should be provided with a portable air pump. *N. mercedis* is separated from the other organisms and any specimen injured or does not appear to be in good condition is discarded. Specimens can be picked up using a bulb pipette with a 5-mm bore or with a plastic spoon. An alternate collecting method is towing a plankton net (0.5-mm mesh) from a boat in the open water. This technique can result in high mysid mortality and much accompanying detritus. *N. mercedis* are abundant between February and July but scarce the remainder of the year **(41)**.

X2.2.1 The following laboratory procedures are based largely on the most current information available from the California Department of Fish and Game Aquatic Toxicology Laboratory.[7] *N. mercedis* can be maintained in static 75 to 114-L aquaria supplied with aeration and a subsurface filter of dolomite 3 to 5 cm in thickness. *N. mercedis* is extremely sensitive to nitrogenous wastes and aquaria should be cleaned daily to remove excess food. A flow-through system supplied with sufficient water for a minimum of two tank volumes per day has also been successful. Successful cultures have been maintained at a temperature of between 15 and 19°C (optimum 17°C), hard fresh water (150 to 200 mg/L $CaCO_3$, hardness and alkalinity), and additional natural sea water or reconstituted sea water to salinity of 1 to 3 g/kg (optimum 2 g/kg). Mysids should be fed *Artemia salina* nauplii (see Practice E 1203) three times a day at the rate of 50 nauplii/mysid/feeding (a total of 150 nauplii/mysid/day) and an artificial food supplement containing vitamins and minerals (0.02 to 0.06 mg/mysid) every other day. Supplements of rotifers and algae may also be beneficial. Cultures have been successfully maintained at densities of less than 10 mysids/L; densities greater than this often result in high mysid mortality.

X2.3 *Toxicity Test Specifications,* Acute static or flow-through toxicity tests (see Guide E 729) are conducted preferably with young mysids in accordance with other studies with this group (see Guide E 1191 and Ref **(45)**). To collect young mysids for testing, females carrying embryos, that are in the eye-development stage, are placed in brood chambers, 7 to 14 days prior to starting the test. Brood chambers can be cages covered with 1.5 to 2.0-mm nytex mesh that allows the neonates to escape into the main body of the aquarium but retains the adults. Neonates are removed each day from the aquarium with a fine mesh dip net (0.5-mm mesh) and transferred to a dish where specimens can be examined for condition and the healthy ones removed for testing. The young released over a two to three-day period should be pooled and transferred to a holding vessel until sufficient numbers are obtained for a test. Ten to 20 specimens are transferred to each test chamber using a pipette with a 5-mm bore. The mysids are fed brine shrimp larvae three times a day at the rate of 30 nauplii/mysid (a total of 90 nauplii/mysid/day) during the test period. The test chambers are examined daily, mortality is recorded, and all dead specimens and debris are removed.

[7] California Department of Fish and Game Aquatic Toxicology Laboratory, 9300 Elk Grove Florin Road, Elk Grove, CA 95624.

X2.4 *Life Cycle and Age Class—Neomysis mercedis* has a short life cycle. Under laboratory conditions and water temperatures of 15 to 19°C, a life cycle is completed in approximately three months. A gravid female will carry an average of 20 embryos in a brood of which an average of seven will be released.

X2.5 *Identification; Systematics of Neomysis(*30*)—N. mercedis* belongs to the awatchensis group of species within the genus. This group is distinguished from the other mysid genera by the acute spiniform apex of the antennal scale (see Fig. X1.14), by the short broad triangular telson with a truncate apex, and by the relatively few distally placed spines along the lateral margins (see Fig. X1.15). Four species have been placed in the awatchensis: *N. awatchensis* **(1)**, *N. intermedia* **(34)**, *N. nigra*, and *N. mercedis* **(33)**.

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