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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 E1463; 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] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

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

E943 Terminology Relating to Biological Effects and Environmental Fate

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1203 Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology

IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

3. Terminology

3.1 The 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 E943).

3.2.2 For definitions of other terms used in this guide, refer to Guide E729, Terminology E943, Guides E1191 and E1192, and Practice E1203. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 The toxicity of a substance in marine or estuarine waters can be determined through a 96-h acute 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**).[3] 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 E1023) or when deriving water quality criteria for aquatic organisms (**2**).

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

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

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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.

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. A15 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.

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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.

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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.

TABLE 2 Quantities of Reagent-Grade[5] Chemicals to Be Added to Aerated Soft Reconstituted Fresh Water to Buffer pH (From Guide E729)

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 E729).

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 E729)

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.

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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 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[4] 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,

[4] "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 Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

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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 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[4] or better and its concentration in any test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient of a mixture, formulation, or commercial product unless an extra amount of solvent is used in 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

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