Designation: E1192 – 97 (Reapproved 2014)

Standard Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians[1]

This standard is issued under the fixed designation E1192; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers procedures for obtaining laboratory data concerning the adverse effects of an aqueous effluent on certain species of freshwater and saltwater fishes, macroinvertebrates, and amphibians, usually during 2 to 4-day exposures, depending on the species, using the static, renewal, and flow-through techniques. These procedures will probably be useful for conducting acute toxicity tests on aqueous effluents with many other aquatic species, although modifications might be necessary.

1.2 Other 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. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting acute toxicity tests on aqueous effluents.

1.3 This guide is based in large part on Guide E729. The major differences between the two guides are (*1*) the maximum test concentration is 100 % effluent or ambient sample, (*2*) testing is not chemical specific, and (*3*) the holding time of effluent and ambient samples is often considerably less than that for chemicals and other test materials. Because the sample is often a complex mixture of chemicals, analytical tests cannot generally be used to confirm exposure concentrations.

1.4 Selection of the technique to be used in a specific situation will depend upon the needs of the investigator and upon available resources. Static tests provide the most easily obtained measure of acute toxicity, but should not last longer than 48 h. Renewal and flow-through tests may last longer than 48 h because the pH and concentrations of dissolved oxygen and effluent are maintained at desired levels and degradation and metabolic products are removed. Static tests might not be applicable to effluents that have a high oxygen demand, or contain materials that (*1*) are highly volatile, (*2*) are rapidly biologically or chemically transformed in aqueous solutions, or (*3*) are removed from test solutions in substantial quantities by the test chambers or organisms during the test. Flow-through tests are generally preferable to renewal tests, although in some situations a renewal test might be more cost-effective than a flow-through test.

1.5 In the development of these procedures, an attempt was made to balance scientific and practical considerations and to ensure that the results will be sufficiently accurate and precise for the applications for which they are commonly used. A major consideration was that the common uses of the results of acute tests on effluents do not require or justify stricter requirements than those set forth in this guide. Although the tests may be improved by using more organisms, longer acclimation times, and so forth, the requirements presented in this guide should usually be sufficient.

1.6 Results of acute toxicity tests should usually be reported in terms of a median lethal concentration (LC50) or median effective concentration (EC50). In some situations, it might be necessary only to determine whether a specific concentration is acutely toxic to the test species or whether the LC50 or EC50 is above or below a specific concentration.

1.7 This guide is arranged as follows:

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

Current edition approved Oct. 1, 2014. Published December 2014. Originally approved in 1988. Last previous edition approved in 2008 as E1192 – 97(2008). DOI: 10.1520/E1192-97R14.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E724 Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs

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

E943 Terminology Relating to Biological Effects and Environmental Fate

E1203 Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology (Withdrawn 2013)[3]

E1604 Guide for Behavioral Testing 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 words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified 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 13.1). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 The term "effluents" refers to aqueous discharges regulated under the National Pollutant Discharge Elimination System (NPDES) collected at the sampling point specified in the NPDES permit.

3.3 The term "ambient samples" refers to water samples collected from the environment. Examples include surface waters, storm waters, leachates, and ground water.

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

4. Summary of Guide

4.1 In each of two or more treatments, test organisms of one species are maintained for 2 to 8 days in one or more test chambers. In each of the one or more control treatments, the organisms are maintained in dilution water to which no effluent has been added in order to provide (*1*) a measure of the acceptability of the test by giving an indication of the quality of the test organisms and the suitability of the dilution water, test conditions, handling procedures, and so forth, and (*2*) the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, the organisms are maintained in dilution water to which a selected concentration of effluent has been added. Data on effects on the organisms in each test chamber are usually obtained periodically during the test and analyzed to determine LC50s or EC50s for various lengths of exposure.

5. Significance and Use

5.1 An acute effluent toxicity test is conducted to obtain information concerning the immediate effects on test organisms of a short-term exposure to an effluent under specific experimental conditions. One can directly examine acute effects of complex mixtures of chemicals as occurs in effluents and some ambient waters. Acute effluent toxicity tests can be used to evaluate the potential for designated-use or aquatic life imperiment in the receiving stream, lake, or estuary. An acute toxicity test does not provide information about whether delayed effects will occur, although a post-exposure observation period, with appropriate feeding if necessary, might provide such information.

5.2 Results of acute effluent tests might be used to predict acute effects likely to occur on aquatic organisms in field

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

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

situations as a result of exposure under comparable conditions, except that (*1*) motile organisms might avoid exposure when possible, (*2*) toxicity to benthic species might be dependent on sorption or settling of components of the effluent onto the substrate, and (*3*) the effluent might physically or chemically interact with the receiving water.

5.3 Results of acute effluent tests might be used to compare the acute sensitivities of different species and the acute toxicities of different effluents, and to study the effects of various environmental factors on results of such tests.

5.4 Acute tests are usually the first step in evaluating the effects of an effluent on aquatic organisms.

5.5 Results of acute effluent tests will depend on the temperature, composition of the dilution water, condition of the test organisms, exposure technique, and other factors.

6. Apparatus

6.1 *Facilities*—Although some small organisms can be held and acclimated in static or renewal systems, most organisms are held, acclimated, and cultured in flow-through systems. Test chambers should be in a constant-temperature room, incubator, or recirculating water bath. A dilution-water tank, which may be used to store receiving water, or a headbox is often elevated so dilution water can be gravity-fed into holding and acclimation tanks and test chambers. Pumps are often used to deliver dilution water and effluent to headboxes and tanks. Strainers and air traps should be included in the water supply. Headboxes and holding, acclimation, culture, and dilution-water tanks should be equipped for temperature control and aeration (see 8.3). Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of air through a 0.22 µm bacterial filter might be desirable (**1**). The facility should be well ventilated and free of fumes. To further reduce the possibility of contamination by components of the effluent and other substances, especially volatile ones, holding, acclimation, and culture tanks should not be in a room in which toxicity tests are conducted, effluent is stored, test solutions are prepared, or equipment is cleaned. During holding, acclimation, culture, and testing, organisms should be shielded from disturbances with curtains or partitions to prevent unnecessary stress. A timing device should be used to provide a 16-h light and 8-h dark photoperiod. A 15 to 30-min transition period (**2**) when the 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 *Special Requirements*—Some organisms require special conditions during holding, acclimation, and testing. For example, burrowing mayfly nymphs should be provided a substrate suitable for burrowing (**3**); immature stream insects should be in a current (**4**); and crabs, shrimp, and bottom-dwelling fish should be provided a silica-sand substrate. Because cannibalism might occur among many species of decapod crustaceans, the claws of crabs and crayfish should be banded, or the individuals should be physically isolated by means of screened compartments.

6.3 *Construction Materials*—Equipment and facilities that contact effluent samples, 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 aquatic organisms. In addition, equipment and facilities that contact effluent samples or test solutions should be chosen to minimize sorption of effluent components 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, acclimation, and culture tanks and in the water supply, but they should be soaked, preferably in flowing dilution water, for a week or more before use (**5**). Cast iron pipe should not be used with salt water and probably should not be used in a freshwater-supply 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 (see Guide E729). Brass, copper, lead, galvanized metal, and natural rubber should not contact dilution water, effluent, or test solutions before or during the test. Items made of neoprene rubber or other materials not mentioned above should not be used unless it has been shown that either (*1*) unfed individuals of a sensitive aquatic species (see 8.2.3) 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 that does not contain the item, or (*2*) their use will not adversely affect survival, growth, or reproduction of a sensitive species.

6.4 *Metering System:*

6.4.1 For flow-through tests, the metering system should be designed to accommodate the type and concentration(s) of the effluent and the necessary flow rates of test solutions. The system should mix the effluent with the dilution water immediately before they enter the test chambers and reproducibly (see 6.4.4) supply the selected concentration(s) of effluent (see 9.5). 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 effluent in, and the flow rates of, test solutions (see Guide E729).

6.4.2 The following factors should be considered when selecting a metering system: (*1*) the installation and use of the apparatus in a fixed or mobile laboratory; (*2*) availability of adequate space and structural requirements for the system, test chambers, and effluent and dilution water storage; (*3*) the applicability of the metering system to specific effluent characteristics (for example, high suspended solids, volatiles, and so forth.); (*4*) the system's dependability, durability, flexibility, and ease of maintenance and replacement; (*5*) the ability to achieve the necessary precision for both flow rate and concentration; and (*6*) cost.

6.4.3 The metering system should be calibrated before and after the test by determining the flow rate through each test chamber and measuring either the concentration of effluent 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.

6.4.4 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 to provide at least ten volume additions per 24 h, in case (*1*) the loading is high (see 11.4) or (*2*) there might be rapid loss of components of the effluent due to microbial degradation, hydrolysis, oxidation, photolysis, reduction, 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 %.

6.5 *Test Chambers:*

6.5.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. However, screens, cups, and so forth, may be used to create two or more compartments within each chamber. Therefore, the test solution can flow from one compartment to another within a test chamber, but, by definition, cannot flow from one chamber to another. Because solution can flow from one compartment to another in the same test chamber, the temperature, concentration of test material, and levels 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, especially in static and renewal tests, to reduce evaporation of test solution and components of the effluent. Also, chambers filled to within 150 mm of the top sometimes need to be covered to prevent organisms from jumping out. All chambers and compartments in a test must be identical.

6.5.2 Test chambers may be constructed by welding, but not soldering, stainless steel or by gluing double-strength or stronger window glass with clear silicone adhesive. Stoppers and silicone adhesive sorb some organochlorine and organophosphorus pesticides, which are then difficult to remove. Therefore, as few stoppers and as little adhesive as possible should be in contact with test solution. If extra beads of adhesive are needed for strength, they should be on the outside of chambers rather than on the inside. Especially in static and renewal tests, the size and shape of the test chambers might affect the results of tests on effluents that contain components that volatilize or sorb onto the chambers in substantial quantities.

6.5.3 The minimum dimensions of test chambers and the minimum depth of test solution depend on the size of the individual test organisms and the loading (see 11.4). The smallest horizontal dimension of the test chambers should be at least three times the largest horizontal dimension of the largest test organism. The depth of the test solution should be at least three times the height of the largest test organism. In addition, the test solution should be at least 150-mm deep for organisms over 0.5 g (wet weight) each, and at least 50-mm deep for smaller organisms. Use of excessively large volumes of solution in test chambers will probably unnecessarily increase the amount of dilution water and effluent used, and, in flow-through tests, increase the average retention time.

6.5.4 For static and renewal tests, organisms weighing more than 0.5 g each (wet weight) are often exposed in 19-L (5-gal) wide-mouth soft-glass bottles containing 15 L of solution or in 300 by 600 by 300-mm deep all-glass test chambers. Smaller organisms are often exposed in 3.8-L (1-gal) wide-mouth soft-glass bottles or battery jars containing 2 to 3 L of solution. Daphnids and midge larvae are often exposed in 250-mL beakers containing 150 to 200 mL of solution.

6.5.5 For flow-through tests, chambers may be constructed by modifying glass bottles, battery jars, or beakers to provide screened overflow holes, standpipes, or V-shaped notches. Organisms weighing more than 0.5 g each (wet weight) are often exposed in 30 L of solution in 300 by 600 by 300-mm deep all-glass test chambers. Smaller organisms are often exposed in 2 to 4 L of solution. In tests with daphnids and other small species, the test chambers or metering system, or both, should be constructed so that the organisms are not stressed by turbulence (**6**).

6.5.6 Embryos are often exposed in glass cups with stainless steel or nylon-screen bottoms or cups constructed by welding stainless steel screen or gluing nylon screen with clear silicone adhesive. The cups should be suspended in the test chambers so as to ensure that the embryos are always submerged and that test solution regularly flows into and out of the cups without creating too much turbulence.

6.6 *Cleaning*—The metering system, test chambers, and equipment used to prepare and store dilution water, effluent, and test solutions should be cleaned before use. New items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid (HCl)), and at least twice with deionized, distilled, or dilution water. (Some lots of organic solvents might leave a film that is insoluble in water.) A dichromate-sulfuric acid cleaning solution may be used in place of both the organic solvent and the acid, but it might attack silicone adhesive. At the end of the test, all items that will be used again should be immediately (*1*) emptied, (*2*) rinsed with water, (*3*) cleaned by a procedure appropriate for removing known components of the effluent (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), and (*4*) rinsed at least twice with deionized, distilled, or dilution water. Acid is often used to remove mineral deposits, and 200 mg of hypochlorite (ClO^-)/L is often used to remove organic matter and for disinfection. (A solution containing about 200 mg ClO^-/L may be prepared by adding 6 mL of liquid household chlorine bleach to 1 L of water. However, hypochlorite is quite toxic to many aquatic animals (**7**) 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 (see 8.2.3) 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 hypochlorite.) The metering system and test chambers should be rinsed with dilution water just before use.

6.7 *Acceptability*—The acceptability of new holding, acclimation, and testing facilities should be demonstrated with a sensitive species (see 8.2.3) before use.

7. Hazards

7.1 Many materials can adversely affect humans if precautions are inadequate. Therefore, skin contact with all effluents and solutions should be minimized by wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses, and by using dip nets, forceps, or tubes to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on effluents containing volatile materials. Information on toxicity to humans (**8**),[4] recommended handling procedures (**9**), and chemical and physical properties of components of the effluent should be studied before a test is begun. Special procedures might be necessary with effluents that contain materials that are radioactive (**10**), or are, or might be, carcinogenic (**11**).

7.2 Although disposal of effluent, 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. Treatment of effluent and test solutions might be desirable before disposal.

7.3 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.

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

7.5 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.

7.6 Because dilution water and test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help prevent electrical shocks. Salt water is such a good conductor that protective devices are strongly recommended.

7.7 To protect hands from being cut by sharp edges of shells, cotton work gloves should be worn (over appropriate protective gloves (see 7.1) if necessary) when juvenile and adult bivalve molluscs are handled.

7.8 Personnel who will be handling an effluent or solutions of it should discuss the advisability of immunization shots with medical personnel and should wash immediately after coming in contact with effluent or test solutions.

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

8. Dilution Water

8.1 *Requirements*—Besides being available in adequate supply, the dilution water should be acceptable to the test organisms and the purpose of the test. The minimal requirement for an acceptable dilution water for acute toxicity tests is that healthy organisms survive in it through acclimation and testing without showing signs of stress, such as discoloration, unusual behavior, or death. A better criterion for an acceptable dilution water is that at least one species of aquatic animal (preferably the one being tested or one taxonomically similar) can survive, grow, and reproduce satisfactorily in it.

8.2 *Source:*

8.2.1 The dilution water for effluent toxicity tests should be a representative sample of the receiving water obtained as close to the point of discharge as possible but upstream of or outside the zone of influence of the effluent. Other factors, such as possible toxicity, eutrophication, and indigeneous food should be considered in selecting a collecting site. The dilution water should be obtained from the receiving water as close to the start of the test as practical but never more than 96 h prior to the beginning of the test. If the receiving water contains effluent from one or more other dischargers, it might be desirable to collect dilution water further upstream or further away from the point of discharge either in addition to or as an alternative to the receiving water. When a test is conducted on effluent being discharged into an estuary, it might be more practical to transport the dilution water to the test facility. Dilution water is often collected from an estuary at slack high tide, but this might contain effluent that was backwashed upstream during the incoming tide. Therefore, it might be preferable to collect the dilution water on the outgoing tide close to, but upstream of, the mixing zone.

8.2.2 If an acceptable dilution water cannot be obtained from the receiving water, an uncontaminated, well-aerated surface or ground water with hardness or salinity within 10 % and pH within 0.2 units of those of the receiving water at the time of the test may be used. It is also desirable that the alkalinity and conductivity be within 25 % of those of the receiving water at the time of the test. If a reconstituted water is used for the dilution water, procedures for preparing the water should be carefully followed (see Guide E729).

8.2.3 Chlorinated water should not be used as, or in the preparation of, dilution water because residual chlorine and chlorine-produced oxidants are quite toxic to many aquatic animals (**7**). Dechlorinated water should be used only as a last resort because dechlorination is often incomplete. Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines (**12**). Some organic chloramines, however, react slowly with sodium bisulfite (**13**). In addition to residual chlorine, municipal drinking water often contains unacceptably high concentrations of copper, lead, zinc, and fluoride, and quality is often rather variable. Excessive concentrations of most metals can usually be removed with a chelating resin (**14**), 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 at least three times each week on nonconsecutive days

that in fresh samples of dilution water either (*1*) *Acartia tonsa*, mysids (less than 24-h post-release from the brood sac), bivalve mollusc larvae, or daphnids (less than 24-h old) 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 (*2*) 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 6.5 µg/L (**7**).

8.2.4 When dilution water is to be transported to the test facility, one or more tanks of adequate capacity may be filled daily. With highly toxic effluents requiring very large volumes of dilution water to produce the desired test concentrations, it might be convenient to conduct the test near the source of dilution water and transport the effluent.

8.2.5 In some situations the selected dilution water might adversely affect the test organisms. Therefore it is sometimes desirable to include a performance control in the test, that is, to maintain organisms during the test in the water from which they were obtained in order to determine whether any effects seen in the dilution-water control were due to the quality of the water or the quality of the organisms.

8.3 *Treatment:*

8.3.1 Dilution water may be aerated by such means as air stones, surface aerators, or column aerators (**15**), (**16**) prior to addition of the effluent. Adequate aeration will bring the pH and concentrations of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in dilution water should be between 90 and 100 % of saturation (**17**) to help ensure that dissolved oxygen concentrations are acceptable in test chambers. Supersaturation by dissolved gases, which might be caused by heating the dilution water, should be avoided to prevent gas bubble disease (**16**), (**18**).

8.3.2 Dilution water may be filtered through a noncontaminating (for example, nylon) sieve with 2-mm or larger holes to remove debris and break up large floating or suspended solids. If necessary, dilution water may be filtered through a sieve with smaller holes (for example, 35 µm is sufficiently small) to remove parasites and predatory organisms if the test organisms are small.

8.3.3 When toxicity tests are conducted with saltwater species, the freshwater component of an effluent might cause an additional stress just as would an extreme pH. Similarly, an effluent with a high salt content might cause an additional stress in tests with freshwater species. In order to measure the whole impact of the effluent, the salinity of the effluent should not be adjusted and the salinity of dilution water should be equal to that of the receiving water outside the zone of influence of the effluent. This same dilution water without the addition of effluent should be used in the dilution-water control treatment. If it is desired to determine the toxicity of the effluent in the absence of any stress due to high or low salinity, the salinity of the effluent or the dilution water, or both, may be adjusted. Adjustment of the salinity of the effluent might affect the toxicity of the effluent. As an alternative to adjusting salinity, it might be desirable to conduct a test with a species that can tolerate both fresh and salt water.

8.4 *Characterization*—The following items should be measured on each batch of dilution water (or daily if dilution water is pumped continuously from a surface water source):

8.4.1 *Fresh Water*—Hardness, alkalinity, conductivity, pH, particulate matter, and total organic carbon.

8.4.2 *Salt Water*—Salinity or chlorinity, pH, particulate matter, and total organic carbon.

8.4.3 For each analytical method used (see 12.2) the detection limit should be below the concentration in the dilution water.

9. Effluent

9.1 *Sampling Point*—The effluent sampling point should be the same as that specified in the National Pollutant Discharge Elimination System (NPDES) permit if the test is conducted for NPDES monitoring purposes (**19**). In some cases, a sampling point between first treatment and the discharge point might provide much better access. If the treated waste is chlorinated, it might be desirable to have sampling points both upstream and downstream of the chlorine contact point to determine the toxicity of both chlorinated and unchlorinated effluent. The schedule of effluent sampling should be based on an understanding of the short- and long-term operations and schedules of the discharger. Although it is usually desirable to evaluate an effluent sample that most clearly represents the normal or typical discharge, conducting tests on atypical samples might also be informative.

9.2 *Collection:*

9.2.1 Several different methods may be used to collect effluent samples for toxicity tests. However, a specific sampling method is frequently specified in the NPDES permit. Selection of a method should be based on the type of test that is to be conducted and the characteristics of the effluent.

9.2.2 Ambient samples may be collected using a variety of methods, depending on the nature of the source. For example, flow proportional sampling is often appropriate for collection of storm water run-off; grab samples might be adequate for pond samples; title estuaries might be sampled using a composite sample.

9.2.2.1 Regardless of the sampling technique employed, effluent samples should be used for testing within 36 h after the end of the collection period, unless it has been shown that toxicity does not change with time.

9.2.3 Flow-through toxicity tests should generally be conducted on effluent obtained by the following methods:

9.2.4 In most cases, continuous, composite, or grab sampling as described above will be suitable. In some cases (such as storm water run-off events or in ambient sample collection) flow-proportional sampling might be most appropriate. It is recommended that provision be made for cooling samples to 4°C during the collection of composite samples. In some cases, flow-proportional sampling might be desirable. Such situations will be governed by the effect of significant flow variation on the retention time of the effluent, and in turn, the effect of altered retention time on loss of components of the effluent. Generally, losses will occur either (*1*) in a treatment basin, or

(*2*) due to hydrolysis or other naturally occurring phenomenon. Flow-proportional sampling, therefore, is recommended only when the variation in flow has a substantial effect relative to these factors. Other sampling techniques are described in detail by Shelley (**19**).

9.3 *Preservation*—If samples are not used within approximately 2 h of collection, they should be preserved by storing them in the dark at about 4°C.

9.4 *Treatment*—Except as per 8.3.3, the sample of effluent must not be altered except that it may be filtered through a nylon (or comparable) sieve or screen with 2-mm or larger holes. Undissolved materials should be uniformly dispersed by gentle agitation immediately before any sample of effluent is distributed to test chambers.

9.5 *Test Concentration(s):*

9.5.1 If the test is intended to allow calculation of an LC50 or EC50, the test concentrations (see 11.1.1.1) should bracket the predicted LC50 or EC50. A prediction might be based on the results of a test on the same or a similar effluent with the same or a similar species. If a useful prediction is not available, it is usually desirable to include additional lower effluent concentrations in the design to ensure bracketing of the LC50.

9.5.2 In some situations (usually regulatory), it is only necessary to determine (*1*) whether a specific concentration is acutely toxic to the test species or (*2*) whether the LC50 or EC50 is above or below a specific concentration. For example, the specific concentration might be a concentration specified by a regulatory agency. 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 or EC50.

10. Test Organisms

10.1 *Species*—For many effluent and ambient water tests the test species is recommended by a regulatory agency. Whenever possible, effluent tests should be conducted with a sensitive, important species indigenous to or regularly stocked into the receiving water. However, species sensitivity will depend on the receiving water, the composition of the effluent, and so forth, and is, therefore, generally difficult to determine without conducting tests with a variety of species. If the objective of the test is to determine the site-specific toxicity of an effluent or ambient sample, tests are usually conducted with a readily available, commercially, or recreationally important indigenous species (see Guide E729). The species used should be identified using an appropriate taxonomic key.

10.2 *Age*—All organisms in a test should be uniform in age and size.

10.2.1 *Fish*—Use of fish weighing between 0.1 and 5.0 g each is usually desirable. Unless data on another life stage are specifically desired, tests should be conducted with juvenile fish, that is, postlarval or older and actively feeding, but not sexually mature, spawning, or spent. Tests may be conducted with newly hatched fish, which are sometimes more sensitive than older stages, and embryos if appropriate precautions are taken. All fish in a test should be from the same year class, and the standard (tip of snout to end of caudal peduncle), fork, or total length of the longest fish should be no more than twice that of the shortest fish.

10.2.2 *Invertebrates*—Immature organisms should be used whenever possible, because they are often more sensitive than older individuals of the same species. Among freshwater invertebrates, daphnids should be less than 24-h old; amphipods, mayflies, and stoneflies in an early instar; and midges in the second or third instar. The term "daphnid" refers to all species in the family Daphnidae. Saltwater mysids should be less than 24-h post-release from the brood sac. Ovigerous decapod crustaceans and polychaetes with visible developing eggs in the coelom should not be used.

10.2.3 *Amphibians*—Young larvae should be used whenever possible.

10.3 *Source*—All organisms in a test should be from the same source, because organisms of the same species from different sources might have different acute sensitivities.

10.3.1 Although effluent tests should be conducted with a species that is indigenous to or stocked into the receiving water, the test organisms do not have to be taken from the receiving water. It is often difficult to obtain organisms of the desired age and in good condition from the receiving water, and sometimes collecting permits are difficult to obtain. Also, it is often difficult to determine whether or not motile organisms collected from the receiving water have been previously exposed to the effluent. Some macroinvertebrates and fishes can be cultivated in the laboratory (see Guide E729). Usual sources of other freshwater fishes are commercial, state, and federal hatcheries. Whenever salmon or trout are to be used, they should be obtained from a hatchery that has been certified disease free, for example, free of infectious pancreatic necrosis, furunculosis, kidney disease, enteric redmouth, and whirling disease. Requirements for certification vary from state to state and from species to species. Other species are usually obtained directly from wild populations in relatively unpolluted areas. Importing and collecting permits might be required by local and state agencies. Organisms captured by electroshocking, chemical treatment, and gill nets should not be used.

10.4 *Care and Handling*—Organisms should be cared for and handled properly (**20**) so they are not unnecessarily stressed.

10.4.1 Whenever aquatic animals are brought into a facility, they should be quarantined (*1*) until used or (*2*) for 14 days or until they appear to be disease free, whichever is longer. Dip nets, brushes, other equipment, organisms, or water should not be transferred from a quarantined tank to any other tank without being autoclaved in distilled water or sterilized.

10.4.2 To maintain aquatic animals in good condition and avoid unnecessary stress, they should not be crowded or subjected to rapid changes in temperature or water quality. In general, organisms should not be subjected to more than a 3°C change in water temperature in any 12-h period, and preferably not more than 3°C in 72 h. The concentration of dissolved oxygen should be maintained between 60 and 100 % of saturation (**17**) and continuous gentle aeration is usually desirable. Supersaturation by dissolved gases should be avoided to prevent gas bubble disease (**16**), (**18**).

10.4.3 Holding and acclimation tanks should be scraped or brushed as needed. Between use with different groups of test organisms, tanks should be sterilized by autoclaving or by treatment with an iodophor (**21**) or with 200 mg of hypochlorite/L for at least 1 h, brushed well once during the hour, and then rinsed well. Although iodophors are not very acutely toxic to aquatic animals, hypochlorite is (see 6.6 concerning preparation and removal of hypochlorite).

10.4.4 Organisms should be handled as little as possible. When handling is necessary, it should be done carefully, gently, and quickly so that organisms are not unnecessarily stressed. Organisms that are injured or dropped during handling and fish that touch dry surfaces should be discarded. Glass tubes with rubber bulbs and smooth ends are best for handling small organisms, whereas dip nets are best for handling organisms over 0.5 g each. Such nets are commercially available, or may be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar knotless material. Nets coated with urethane resin are best for handling catfish. Equipment used to handle aquatic organisms should be sterilized between uses (see 10.4.3). Hands should be washed before handling or feeding test organisms.

10.4.5 Organisms should be carefully observed during quarantine, holding, and acclimation for signs of stress, physical damage, mortality, disease, and external parasites. Abnormal, dead, and injured individuals should be discarded. If visual examination of the behavior and external appearance of test organisms indicates that they are not eating or are flashing, flipping, swimming erratically, emaciated, gasping at the surface, hyperventilating, hemorrhaging, producing excessive mucus, or showing abnormal color, the cause should be determined and eliminated. If organisms show signs of disease or external parasites, appropriate action should be taken (see 10.6).

10.5 *Feeding*—At least once a day, organisms should be fed a food that will support normal function. Live brine shrimp nauplii (see Practice E1203) are a good food for many aquatic species.

10.6 *Disease Treatment*—Fish may be chemically treated to cure or prevent some diseases using appropriate treatments (see Guide E729). Severely diseased fish and all other diseased animals should be discarded immediately, because systemic bacterial infections usually cannot be treated effectively, internal parasites cannot be removed without extensive treatment, viral diseases cannot be treated, and diseased invertebrates can rarely be treated effectively. Generally, organisms should not be treated during the first 16 h after arrival at the test facility because of possible stress or drug treatment during collection or transportation. However, immediate treatment is necessary in some situations, such as treatment of bluegills for columnaris disease during hot weather. Tests must not be begun with treated organisms for at least 4 days after treatment, and organisms must not be treated during the test.

10.7 *Holding*—Small organisms may be held in aerated, constant-temperature static or renewal systems. Most species, however, should be held in uncontaminated, aerated water of constant temperature and quality in a flow-through system with a flow rate of at least two volume additions per day. When possible, the organisms should be held in the dilution water and at the temperature at which they are to be tested. During long holding periods, however, it is generally easier and safer to hold fish at temperatures lower than those given in Guide E729 because the metabolic rate and the number and severity of disease outbreaks are reduced.

10.8 *Acclimation:*

10.8.1 Except for species that should be less than 48 h old at the beginning of the test, the test organisms should be slowly introduced to the dilution water and test temperature by gradually changing from the water they were in to 100 % dilution water over a period of 24 h or more and changing the water temperature at a rate not to exceed 3°C within 12 h, and preferably not to exceed 3°C in 72 h. They should be maintained in the dilution water at the test temperature for at least the last 24 h before they are placed in test chambers to ensure that the test organisms are in reasonably acceptable condition. Complete acclimation, which has not been adequately experimentally defined, might take considerably longer; therefore, organisms should be maintained in the dilution water at the test temperature for more than 24 h whenever possible.

10.8.2 Young amphibian larvae and fish that have been actively feeding for less than about 20 days, freshwater amphipods, daphnids, midge larvae, and saltwater mysids must be fed, and all other insects may be fed, up to the beginning of the test. All other amphibian larvae and fish over 0.5 g each must not be fed for 48 h, and all other invertebrates over 0.5 g each must not be fed for 24 h, before the beginning of the test. If adult amphipods or daphnids are isolated before the beginning of the test for the collection of young, the adults must be fed.

10.9 *Quality:*

10.9.1 A group of organisms should not be used for a test if more than about 10 % of the individuals show signs of disease or stress, such as discoloration, unusual behavior, or death during the 24 h immediately preceding the test. If the percentage is greater than about 10, all individuals should be either discarded or treated, held an additional 4 days, and reacclimated if necessary.

10.9.2 Reference toxicants might be useful for assessing the quality of test organisms. Many chemicals have been used or evaluated as reference toxicants (see Guide E729), but none has been proven to be a reliable indicator of the overall quality of any species or test results. A reference toxicant is more likely to be useful when used in conjunction with tests on materials that have the same mode of action as the reference toxicant.

11. Procedure

11.1 *Experimental Design:*

11.1.1 Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and numbers of test chambers and organisms per treatment should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 14). One of

the following two types of experimental design will probably be appropriate in most cases.

11.1.1.1 An acute effluent test intended to allow calculation of an LC50 or EC50 usually consists of one or more control treatment(s) (see 8.2.5) and a geometric series of at least five concentrations of the effluent. In the control treatment(s), organisms are exposed to dilution water to which no effluent has been added. Except for the control(s) and the highest concentration, each concentration should be at least 50 % 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 0.5, five properly chosen concentrations will often provide LC50s or EC50s for several durations and are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of acute toxicity is particularly nebulous (see 9.5.1), six or seven concentrations might be desirable.

11.1.1.2 If it is only necessary to determine (*1*) whether a specific concentration is acutely toxic to the test species or (*2*) whether the LC50 or EC50 is above or below a specific concentration (see 9.5.2), only that concentration and the control(s) are necessary. Two additional concentrations at about one half and two times the specific concentration of concern are desirable to increase confidence in the results.

11.1.1.3 If an LC or EC near the extremes of toxicity, such as an LC5 or LC95, is to be calculated, at least one concentration of effluent should have killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the LC or EC is to be calculated. This requirement might be met in a test to determine an LC50 or EC50, but special tests with appropriate test concentrations and more test organisms per treatment will usually be necessary. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are normally more reliable than reporting a calculated result such as an LC5 or LC95 unless several percent killed or affected were obtained close to 5 or 95 %.

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, which is defined as the smallest physical entity to which treatments can be independently assigned. Because test solution can flow from one compartment to another, but not from one test chamber to another (see 6.5.1), the test chamber is the experimental unit. As the number of test chambers (that is, experimental units) per treatment increases, the number of degrees of freedom 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 practical. For example, the temperature in all test chambers should be as similar as practical 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 reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or rectangle) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of test chambers and organisms per treatment should be calculated from (*1*) the expected variance within test chambers, (*2*) the expected variance between test chambers within a treatment, and (*3*) the maximum acceptable width of the confidence interval on the LC50 or EC50 (**22**). If such calculations are not made, at least 10 organisms should be exposed to each treatment in static and renewal tests, and at least 20 organisms in flow-through tests. If each test concentration is more than 50 % of the next higher one, fewer organisms per concentration of effluent, but not the control treatment(s), may be used. Organisms in a treatment should be divided between two or more test chambers in order to allow estimation of experimental variation (**23**). If the controls are important in the calculation of results (possibly because of correction for spontaneous mortality using Abbott's formula), it might be desirable to use more test chambers and test organisms for the control treatment(s) than for each of the other treatments.

11.2 *Dissolved Oxygen:*

11.2.1 The dissolved oxygen concentration in each test chamber should be between 40 and 100 % of saturation (**17**) at all times during the test.

11.2.2 If the concentration of dissolved oxygen and oxygen demand in any test solution at the beginning of the test are such that the concentration of dissolved oxygen in the test solution during the test will probably fall below 40 % of saturation even if no test organisms are present, the test solutions may be gently aerated during the test. Turbulence, however, should be avoided because it might stress test organisms, re-suspend 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 or if the dissolved oxygen concentration will probably fall below 40 % of saturation, it might be desirable to conduct simultaneous tests with and without aeration to determine if aeration affects the results of the test.

11.3 *Temperature:*

11.3.1 For constant-temperature static tests, the difference between the highest and lowest temperature measured during the test should not exceed 4°C. The test temperature should be that measured at the surface of the receiving water just upstream from the outfall at noon (local time) on the first day of acclimation or testing, because the temperature at noon usually approximates the average temperature for the day. If more practical, however, the test temperature may be that at which the test organisms were held prior to transportation to the testing site. Static tests may also be conducted at fluctuating temperature, such as by pumping receiving water through a water bath in which the test chambers are located.

11.3.2 For flow-through tests the actual test temperature may be relatively constant (±2°C) or may fluctuate between the mean daily maximum and minimum temperatures of the receiving water measured at the surface just outside the zone of influence of the effluent at the time of the test. Temperature can be controlled by passing effluent or dilution water, or both, through separate stainless steel coils immersed in a heating or cooling water bath prior to entering the test chambers. Because the temperature of industrial effluents is sometimes higher than that at which the test organisms are acclimated, it is important to have the capability of lowering the temperature.

11.3.3 Selection of the test temperature should take into account the type of species and the characteristics of the body of water. For example, in some situations the temperature at the surface is substantially higher than the temperature to which benthic species are exposed, and fish might avoid extreme temperatures when possible.

11.4 *Loading:*

11.4.1 The grams of organism (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, loading should be limited to ensure that (*1*) the concentration of dissolved oxygen does not become unacceptably low and (*2*) the test organisms are not stressed because of aggression or crowding.

11.4.2 In static and renewal tests, the loading in each test chamber should not exceed 0.8 g/L at any time. The loading should not exceed 0.5 g/L if the test temperature is above the temperature suggested for the species in Guide E729 and at all temperatures above 17°C. If necessary, a lower loading should be used to keep the concentration of dissolved oxygen from falling below 40 % of saturation (see 11.2) in any chamber containing live test organisms.

11.4.3 In flow-through tests, the loading in each test chamber should not exceed 10 g/L at any time in any test chamber and should not exceed 1 g/L of solution passing through the chamber in 24 h. The loading should not exceed 5.0 g/L in the chambers or 0.5 g/(L/day) if the test temperature is higher than the temperature suggested for the species in Guide E729 and at all temperatures higher than 17°C. If necessary, higher flow rates or lower loadings, or both, should be used to maintain the concentration of dissolved oxygen above 40 % of saturation (see 11.2) in any chamber containing live test organisms.

11.4.4 A lower loading should be used if aggression occurs.

11.4.5 Comparable loadings should be used for other species.

11.5 *Beginning the Test:*

11.5.1 A representative sample of the test organisms must be either (*1*) impartially 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 (*2*) assigned either by random assignment of one organism to each chamber, random assignment of a second organism to each chamber, and so forth, or by total randomization. It is often convenient to assign organisms to other containers, and then add them to the test chambers all at once. Caution should be exercised to minimize the transfer of dilution or culture water with the test organisms to the chambers, particularly in higher effluent treatments, ambient water treatments, and for tests using small test volumes.

11.5.2 The test begins when the test organisms are first exposed to the effluent.

11.5.3 Static and renewal tests should be begun by placing test organisms in the chambers within 30 min after the effluent was added to the dilution water.

11.5.4 Flow-through tests should be begun by either (*1*) placing test organisms in the chambers after the test solutions have been flowing through the chambers long enough for the concentrations of effluent to have reached steady state, or (*2*) 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 effluent sufficient to establish the desired test concentration in the test chamber at the time of activation of the metering device. Alternative (*1*) allows the investigator to study the properties of the effluent and the operation of the metering system immediately prior to the test, whereas alternative (*2*) allows the organisms to partially adjust to the chambers before the beginning of the test.

11.6 *Feeding*—Organisms should not be fed during an acute toxicity test or for a time before the test when possible (see 10.8.2), because fecal matter and uneaten food will decrease the dissolved oxygen concentration and the biological activity of some test materials. These problems are most severe with the static technique, but are sometimes important with the renewal and flow-through techniques. If cannibalistic organisms cannot be physically restrained or separated, minimal feeding is necessary. Because saltwater mysids less than 24-h post-release from the brood sac are severely stressed if not fed within 48 h, they should be fed before and during acute tests.

11.7 *Duration of Test*—Daphnids and midge larvae should be exposed to the effluent for 48 h. All other species should be exposed for 96 h in static tests and for at least 96 h in renewal and flow-through tests.

11.8 *Biological Data:*

11.8.1 Death is the adverse effect most often used for the calculation of results of acute toxicity tests with aquatic organisms. The criteria for death are usually lack of movement, especially the absence of respiratory movements in fish and shrimp, and lack of reaction to gentle prodding. Because death of some invertebrates is not easily distinguished from immobilization, an EC50 is usually determined rather than an LC50. For daphnids and midge larvae the EC50 should be based on death plus immobilization, defined as the lack of movement except for minor spontaneous, random activity of appendages. For crabs, crayfish, and shrimp the EC50 should be based on death plus immobilization, defined as lack of movement and lack of response to gentle prodding. Because juvenile and adult bivalve molluscs can close their valves for extended periods of time, acute lethality tests should not be conducted with them. An EC50 based on death plus incomplete shell development can be determined with bivalve mollusc larvae, but special procedures must be used (see Guide E724). In order to account for the total severe acute adverse impact of

the effluent on the test organisms, it is desirable to calculate an EC50 based on death plus immobilization plus loss of equilibrium, defined as the inability to make coordinated movement and maintain a normal upright position. Other effects, such as behavior (see Guide E1604), can be used to determine an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, excitability, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, hemorrhaging, molting, cessation of burrowing by crabs and shrimp, and cannibalism should be reported.

11.8.2 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 tests should be done very gently.

11.8.3 The number of dead and affected 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 and affected 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 so that 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 and in tests with burrowing organisms, 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.

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

11.8.5 Except for such very small organisms as young daphnids and mysids, the weights of the test organisms should be determined by weighing and discarding either (*1*) a representative group of organisms before the test, or (*2*) the control organisms that are alive at the end of the test. For organisms such as adult daphnids and mysids, the dry weight (dried at 60°C for 72 to 96 h or to constant weight) should be measured. The wet weight (blotted dry) of other species should be measured. Except for such species as daphnids and mysids, length should be measured. The standard (see 10.2.1), fork, or total length of fish should be measured.

11.8.6 All organisms used in the test should be destroyed at the end of the test.

11.9 *Other Measurements:*

11.9.1 The pH and concentration of dissolved oxygen should be measured in the control(s) and in the high, medium, and low effluent concentrations at the beginning of the test and every 24 h thereafter as long as live test organisms are in them in the preceeding time observation. Alkalinity, hardness, and conductivity in fresh water and salinity (or chlorinity) in salt water should be measured in the control(s) and the high effluent concentration at the beginning and end of static tests and daily during renewal and flow-through tests.

11.9.2 *Temperature:*

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

11.9.2.2 In static and renewal tests, either (*1*) 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 (*2*) if the test chambers are in a water bath or a constant-temperature room or incubator, the temperature of the water or air must be measured or monitored at least hourly or the maximum and minimum temperatures 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.

11.9.2.3 In flow-through tests, in at least one test 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 the beginning and end of the test, temperature must be measured concurrently in all test chambers.

11.9.3 Although desirable, direct measurement of the concentration of effluent in the test solution(s) is usually not possible. Concentrations usually have to be monitored by such indirect means as measurement of TOC, conductivity, or turbidity or measurement of one or more components of the effluent. Water samples should be taken midway between the top, bottom, and sides of the test containers and should not include any surface scum or material stirred up from the bottom or sides.

11.9.4 Additional measurements on the dilution water, effluent, and test solutions are often desirable.

12. Analytical Methodology

12.1 If samples of dilution water, effluent, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately (**24**) to minimize the loss of components of the effluent by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

12.2 Chemical and physical data should be obtained using appropriate ASTM test methods whenever possible. For those measurements for which ASTM test methods do not exist or are not sensitive enough, methods should be obtained from other reliable sources (**25**). The concentration of un-ionized ammonia may be calculated from the pH, temperature, and concentration of total ammonia (**26**).

12.3 The precision and bias of each analytical method used should be determined using water samples from a control test chamber or brood-stock tank. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

13. Acceptability of Test

13.1 An acute toxicity test on an aqueous effluent should usually be considered unacceptable if one or more of the following occurred, except that, for example, a small difference between test chambers (see 13.1.1) might be inconsequential.

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 dilution-water control was not included in the test.

13.1.4 The test was begun with organisms within 4 days after treatment for a disease or the organisms were treated during the test.

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

13.1.6 More than 10 % of the organisms in the dilution-water control showed signs of disease or stress, such as discoloration, unusual behavior, or death, during the test.

13.2 Calculation of an LC50 or EC50 should usually be considered unacceptable if either or both the following occurred:

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

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

14. Calculation of Results

14.1 Results should be calculated based on the initial volume percent of the effluent in the test solution for static tests, and the average volume percent for renewal and flow-through tests. The volume percent (V) should be calculated using the equation:

$$V = (100 \times V_E)/(V_E + V_D) \quad (1)$$

where:

V_E = Volume of effluent, L, and
V_D = Volume of dilution water, L.

14.2 A variety of methods may be used to calculate an LC50 or EC50, depending on the kind and amount of data obtained from the test (see Guide E729). Whenever an LC or EC is calculated, its 95 % confidence limits should also be calculated.

15. Report

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

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

15.1.2 Source of effluent, date, time, and method of collection, known chemical and physical properties, composition, variability, and a description of any treatment.

15.1.3 Source of dilution water, date, time, and method of collection, known chemical and physical properties, and a description of any treatment.

15.1.4 Source of test organisms, scientific name (and strain for salmonids when appropriate), name of person who identified the organisms and the taxonomic key used, age, life stage, means and ranges of weights and lengths, observed diseases, treatments, holding and acclimation procedures, and food.

15.1.5 Description of the experimental design, test chambers, compartments, and covers, the depth and volume of solution in the chambers, method of beginning the test, numbers of test organisms and chambers per treatment, the loading and lighting, and, for flow-through tests, a description of the metering system and the flow rate as volume additions per 24 h.

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

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

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

15.1.9 Methods used for, and results (with standard deviations or confidence limits) of, chemical analyses of water quality and concentration of effluent, including validation studies and reagent blanks.

15.1.10 Definition(s) of the effect(s) used for calculating LC50s or EC50s and a summary of general observations on other effects.

15.1.11 Table of data on the number of test organisms exposed and killed or otherwise affected 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 For daphnids and midge larvae, the 24- and 48-h, and for all other species, the 24-, 48-, and 96-h LC50s or EC50s and their 95 % confidence limits, and the method used to calculate them; for flow-through tests, enough other LC50s or EC50s to define the shape of the toxicity curve; the highest concentration of effluent that killed or affected no greater a percentage of the test organisms than did the control treatment.

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; ambient samples; amphibians; EC50; effluents; freshwater fishes; freshwater invertebrates; LC50; saltwater fishes; saltwater invertebrates

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