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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:

[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.

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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 (**5**). 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 (**6**) 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 (**7**); immature stream insects should be in a current (**8**); 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 (**9**). 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 (**10**).

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 (**11**) 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 (**1**),[4] recommended handling procedures (**2**), 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 (**3**), or are, or might be, carcinogenic (**4**).

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 (**11**). 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 (**11**).

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