Designation: E 1415 – 91 (Reapproved 1998)

Standard Guide for Conducting Static Toxicity Tests With *Lemna gibba* G3[1]

This standard is issued under the fixed designation E 1415; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the adverse effects of a text material added to growth medium on a certain species of duckweed (*Lemna gibba* G3) during a 7-day exposure using the static technique. These procedures will probably be useful for conducting toxicity tests with other species of duckweed and other floating vascular plants, although modifications might be necessary.

1.2 Special needs or circumstances might also justify modification of this standard. 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 tests with duckweed.

1.3 The procedures in this guide are applicable to most chemicals, either individually or in formulations, commercial products, or known mixtures. With appropriate modifications these procedures can be used to conduct tests on temperature and pH and on such other materials as aqueous effluents (see also Guide E 1192), leachates, oils, particulate matter, sediments and surface waters. These procedures do not specifically address effluents because to date there is little experience using duckweeds in effluent testing and such tests may pose problems with acclimation of the test organisms to the receiving water. Static tests might not be applicable to materials that have a high oxygen demand, are highly volatile, are rapidly biologically or chemically transformed in aqueous solution, or are removed from test solutions in substantial quantities by the test chambers or organisms during the test.

1.4 Results of toxicity tests performed using the procedures in this guide should usually be reported in terms of the 7-day IC50 based on inhibition of growth. In some situations it might only be necessary to determine whether a specific concentration unacceptably affects the growth of the test species or whether the IC50 is above or below a specific concentration. Another end point that may be calculated is the no observed effect concentration (NOEC).

1.5 The sections of this guide appear as follows:

1.6 *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 to determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 6.

2. Referenced Documents

2.1 *ASTM Standards:*

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[2]

E 729 Guide for Conducting Acute Toxicity Tests with

[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

Current edition approved April 15, 1991. Published June 1991.

[2] *Annual Book of ASTM Standards*, Vol 14.02.

Fishes, Macroinvertebrates and Amphibians[3]
E 943 Terminology Relating to Biological Effects and Environmental Fate[3]
E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]
E 1192 Guide for Conducting Acute Toxicity Texts on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[3]
E 1218 Guide for Conducting 96-h Toxicity Tests with Microalgae[3]

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 Section 13). *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*, *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 *Definitions of Terms Specific to This Standard:*

3.2.1 *frond*—individual leaf-like structure on a duckweed plant.

3.2.2 *IC50*—a statistically or graphically estimated concentration of test material that is expected to cause a 50 % inhibition of one or more specified biological processes (such as growth or reproduction), for which the data are not dichotomous, under specified conditions.

3.3 For definitions of other terms used in this guide, refer to Terminology E 943, and Guides E 729 and E 1023. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 In each of two or more treatments, plants of *Lemna gibba* G3 are maintained for 7 days in two or more test chambers using the static technique. In each of the one or more control treatments, the plants are maintained in growth medium to which no test material has been added in order to provide a measure of the acceptability of the test by giving an indication of the quality of the duckweed and the suitability of the growth medium, test conditions, handling procedures, and so forth, and the basis for interpreting data obtained from the other treatments. In each of the one or more other treatments, the duckweed plants are maintained in growth medium to which a selected concentration of test material has been added. Specified data concerning growth of duckweed in each test chamber are obtained during the test and are usually analyzed to determine the IC50 or NOEC based on inhibition of growth.

5. Significance and Use

5.1 The term duckweed commonly refers to members of the family Lemnaceae. This family has many species world-wide in 4 genera. This guide is designed for toxicity testing with one particular clone of one species of duckweed that has been extensively studied, *Lemna gibba* G3, although other species such as *Lemna minor* or *Spirodela* spp. can probably also be tested using the procedures described herein.

5.2 Duckweeds are widespread, free-floating aquatic plants, ranging in the world from tropical to temperate zones. Duckweeds are a source of food for waterfowl and small animals and provide food, shelter, and shade for fish. The plants also serve as physical support for a variety of small invertebrates. Duckweed is fast growing and reproduces rapidly compared with other vascular plants **(1)**.[4] Under conditions favorable for its growth, it can multiply quickly and form a dense mat in lakes, ponds, and canals, primarily in fresh water, but also in estuaries. It also grows well in effluents of wastewater treatment plants and has been suggested as a means of treating wastewaters **(2)**. A dense mat of duckweed can block sunlight and aeration and cause fish kills **(3)**.

5.3 Duckweed is small enough that large laboratory facilities are not necessary, but large enough that effects can be observed visually.

5.4 Because duckweed is a floating macrophyte, it might be particularly susceptible to surface active and hydrophobic chemicals that concentrate at the air-water interface. Results of duckweed tests on such chemicals, therefore, might be substantially different from those obtained with other aquatic species.

5.5 Results of toxicity tests with duckweed might be used to predict effects likely to occur on duckweed in field situations as a result of exposure under comparable conditions.

5.6 Results of tests with duckweed might be used to compare the toxicities of different materials and to study the effects of various environmental factors on results of such tests.

5.7 Results of tests with duckweed might be an important consideration when assessing the hazards of materials to aquatic organism (see Guide E 1023) or when deriving water quality criteria for aquatic organisms **(4)**.

5.8 Results of tests with duckweed might be useful for studying biological availability of, and structure-activity relationships between test materials.

5.9 Results of tests with duckweed will depend on temperature, composition of the growth medium, condition of the test organisms, and other factors. The growth media that are usually used for tests with duckweed contain concentrations of salts, minerals, and nutrients that greatly exceed those in most surface waters.

6. Hazards

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially

[3] *Annual Book of ASTM Standards*, Vol 11.05.

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

when washing equipment or putting hands in test solutions), laboratory coats, aprons, and glasses. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans **(5)**, recommended handling procedures **(6)**, and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radio-labeled test materials **(7)** and with materials that are, or are suspected of being, carcinogenic **(8)**.

6.2 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

6.3 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.

6.4 Acidic solutions and hypochlorite solutions should not be mixed because hazardous fumes might be produced.

6.5 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.

6.6 Because growth medium 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.

7. Apparatus

7.1 *Facilities*—Culture and test chambers should be maintained in an environmental chamber, incubator, or room with constant temperature (see 11.2) and appropriate illumination (see 11.3). A water bath is generally not acceptable because it prevents proper illumination of the test chambers. The facility should be well-ventilated and free of fumes. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, the culture chambers should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned.

7.2 *Test Chambers*—In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which no water connections exist. Glass 250-mL beakers, 200-mL flat-bottomed test tubes, 250-mL fruit jars, and 250 or 500-mL Erlenmeyer flasks have been used successfully **(9-11)**. The ratio of the size of the test chamber to the volume of test solution should be 5 to 2 (that is, 100 mL in a 250-mL Erlenmeyer flask, 200 mL in a 500-mL Erlenmeyer flask). Plastic chambers may be used only if duckweed does not adhere to the walls and the test material does not sorb onto the plastic more than it does to glass. Chambers should be covered to keep out extraneous contaminants and to reduce evaporation of test solution and test material. Beakers should be covered with a clear watch glass and flasks should be covered with loose-fitting caps such as foam plugs, stainless steel caps, Shimadzu enclosures, glass caps or screw caps. (The acceptability of foam plugs should be investigated prior to use because some brands have been found to be toxic.) All chambers and covers in a test must be identical.

7.3 *Cleaning*—Test chambers and equipment used to prepare and store growth medium, stock solutions, 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), and at least twice with deionized or distilled water. (Some lots of some 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. At the end of the test, all items that are to be used again should be immediately (a) emptied, (b) rinsed with water, (c) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), (d) cleaned with a nonphosphate detergent using a stiff bristle brush to loosen any attached materials, and (e) rinsed at least twice with deionized or distilled water. Acid is often used to remove mineral deposits. Chambers should be dried in an oven at 50 to 100°C, capped with appropriate closures, autoclaved for 20 min at 121°C and 1.1 kg/cm^2. Test chambers should be rinsed with growth medium just before use.

7.4 *Acceptability*—Before a toxicity test is conducted with duckweed in new test facilities, it is desirable to conduct a nontoxicant test, in which all test chambers contain growth medium with no added test material, to determine before the first toxicity test whether duckweed will grow acceptably in the new facilities, whether the growth medium, handling procedures, etc., are acceptable, whether there are any location effects on growth, and the magnitudes of the within-chamber and between-chamber variances.

8. Growth Medium

8.1 Growth medium is prepared by adding appropriate amounts of specified reagent-grade[5] chemicals to deionized or distilled water. Recommended growth media are given in Appendix X1.

9. Test Material

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

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material,

9.1.2 Solubility, stability, photodegradability, and volatility in the growth medium,

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

9.1.3 Measured or estimated toxicity to duckweed (if nothing is known about the toxicity to duckweed, a range-finding test is suggested),

9.1.4 Precision and bias of the analytical method at the planned concentration(s) of test material, if the test concentration(s) are to be measured,

9.1.5 Estimate of toxicity to humans, and

9.1.6 Recommended handling procedures (see 6.1).

9.2 *Stock Solution*—In some cases the test material can be added directly to the growth medium, but usually it is dissolved in a solvent to form a stock solution that is then added to growth medium. If a stock solution is prepared, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.1 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is growth medium. Distilled or deionized water may also be used as a solvent, but the amount of water added to growth medium to prepare the test solutions should be kept to less than 10 % of the total volume to avoid dilution of the growth medium. Several techniques have been specifically developed for preparing aqueous stock solutions of slightly soluble materials **(12)**. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such reagents might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols and organic acids, and chloride or nitrate salts of metals, might affect the pH even more than the use of the minimum necessary amount of a strong acid or base.

9.2.2 If a solvent other than growth medium is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect growth of duckweed. Because of its low toxicity to aquatic organisms, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate undesirable growths of microorganisms; acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[5] or better and its concentration in any test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient of a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution.)

9.2.3 If a solvent other than growth medium or water is used, at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test, and a growth medium control should be included in the test. If no solvent other than growth medium or water is used, a growth medium control must be included in the test.

9.2.3.1 If a solvent control is required and the concentration of solvent is the same in all test solutions that contain test material, the solvent control must contain the same concentration of solvent.

9.2.3.2 If a solvent control is required and the concentration of solvent is not the same in all test solutions that contain test material, either (a) a solvent test must be conducted to determine whether growth of duckweed is related to the concentration of the solvent over the range used in the toxicity test or (b) such a solvent test must have already been conducted using the same growth medium. If growth is found to be related to the concentration of solvent, a toxicity test in that medium is unacceptable if any treatment contained a concentration of solvent in that range. If growth is not found to be related to the concentration of solvent, a toxicity test in that same medium may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in all of the other treatments.

9.2.3.3 If the test contains both a growth medium control and a solvent control, the growth of the duckweed in the two controls should be compared using a *t*-test. Adjustments for chamber-to-chamber heterogeneity might be necessary. The use of a large alpha level (for example, 0.25) will make it more difficult to accept the null hypothesis when it should not be accepted. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

9.2.3.4 If a statistically significant difference in growth is detected between the two controls, only the solvent control can be used for meeting the requirements of 13.1.3 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of 13.1.3 and as the basis for calculation of results.

9.2.4 If a solvent other than growth medium or water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of the solvent on the results of the test.

9.3 *Test Concentration(s)*:

9.3.1 If the test is intended to allow calculation of the 7-day IC50, the test concentrations (see 11.1.1.1) should bracket the predicted IC50. A prediction might be based on the results of a test on the same or a similar material with the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which the test species is exposed to a control and three to five concentrations of the test material that differ by a factor of 10. The greater the similarity between the range-finding test and the actual test, the more useful the range-finding test will be.

9.3.1.1 If necessary, concentrations above solubility should be used because organisms in the real world are sometimes exposed to concentrations above solubility and because solubility is often not well known. The use of concentrations that are more than ten times greater than solubility is probably not worthwhile. With some test materials it might be found that concentrations above solubility do not affect growth any more

than does the concentration that is the solubility limit; such information is certainly worth knowing.

9.3.2 In some (usually regulatory) situations, it is only necessary to determine whether a specific concentration of test material unacceptably affects growth of the test species or whether the IC50 is above or below a specific concentration. For example, the specific concentration might be the concentration occurring in a surface water, the concentration resulting from the direct application of the material to a body of water, or the solubility limit of the material in water. When there is only interest in a specific concentration, it is often only necessary to test that specific concentration (see 11.1.1.2), and it is not necessary to actually determine the IC50.

10. Test Organisms

10.1 *Species*—The test species is *Lemna gibba* G3.[6] It is widely distributed, easily handled in the laboratory, and has a history of successful use. The identity of the organism should be verified using an appropriate taxonomic key **(13)**. It is important to identify the clone **(1)**, because it has been shown that different clones of the same species can have different sensitivities **(14)**.

10.2 *Stock Culture*—Plants used in testing must be obtained from laboratory stock cultures that have been actively growing in growth medium under constant warm-white fluorescent illumination of approximately 580 to 620 fc (6200 to 6700 lx) and temperature of 25 ± 2°C for at least the eight weeks immediately preceding the start of the test. Maintenance of axenic stock cultures is recommended. Plants should be aseptically transferred on a regular schedule (weekly is suggested) into fresh growth medium.

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 fronds 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 A test intended to allow calculation of an IC50 usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the medium or solvent controls, or both, (see 9.2.3), duckweed is exposed to growth medium to which no test material has been added. Except for the control(s) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.6, five properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of toxicity is particularly nebulous (see 9.3.1), six or seven concentrations might be desirable.

11.1.1.2 If it is only necessary to determine whether a specific concentration unacceptably affects growth or whether the IC50 is above or below a specific concentration (see 9.3.2), 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 IC near the extremes of toxicity, such as an IC5 or IC95, is to be calculated, at least one concentration of test material should have inhibited growth by a percentage, other than 0 or 100 %, near the percentage for which the IC is to be calculated. This requirement might be met in a test designed to determine an IC50, but a special test with appropriate concentrations of test material will usually be necessary.

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. Thus the test chamber, as defined in 7.2, 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 significant test increases. With respect to factors that might affect results within test chambers and, therefore, results of the test, all test chambers in the test should be treated as similarly as possible. For example, the temperature in all test chambers should be as similar as possible unless the purpose of the test is to study the effect of temperature. Test chambers are usually arranged in one or more rows. Treatments must be randomly assigned to individual test chamber locations and may be randomly reassigned during the test. A randomized block design (with each treatment being present in each block, which may be a row or rectangle) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of test chambers per treatment should be calculated from (a) the expected variance between test chambers within a treatment, and (b) either the maximum acceptable confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing **(15)**. If such calculations are not made, at least three test chambers must be used for each treatment (test concentration and control). If each test concentration is more than 60 % of the next higher one and the results are to be analyzed using regression analysis, fewer test chambers may be used for each treatment that contains test material, but not for the control treatment(s). Replicate test chambers (that is, experimental units) within a treatment are necessary in order to allow estimation of experimental error **(15)**.

11.2 *Temperature*—Tests with *Lemna gibba* G3 should be conducted at 25 ± 2°C. Temperature should be controlled by placing the test chambers in an environmental chamber, incubator, or constant-temperature room. Other temperatures may be used to study the effect of temperature on duckweed or to study the effect of temperature on the toxicity of a material to duckweed.

[6] There is currently no commercial source of *Lemna gibba* G3. It may be available from: Dr. Elaine Tobin, UCLA, Biology Department, Los Angeles, CA 90024, and from Dr. Janet Slovin, USDA, BARC-West, Bldg. 050 HH-4, Beltsville, MD 20705.

11.3 *Illumination*—Continuous warm-white fluorescent lighting should be used to provide a light intensity selected from the range of between 6200 and 6700 lx (580 and 620 fc), as measured adjacent to each test chamber at the surface of the test solution. The light intensity at each position in the incubation area should be measured and should not differ by more than 15 % from the selected light intensity.

11.4 *Beginning the Test*:

11.4.1 A large enough batch of growth medium should be prepared so that (a) the desired volume can be placed in each control test chamber, (b) the necessary volume of each test solution can be prepared, and (c) all desired analyses can be performed (see 11.7). Enough test solution should be prepared for each treatment so that the desired volume can be placed in each test chamber and all desired analyses of water quality, test material, etc. (see 11.7) can be performed.

11.4.2 Uniform, healthy-looking plants should be removed from the stock culture to use in testing. Three to five plants, each consisting of three or four fronds, should be added to each test chamber. Care should be taken to ensure that plants and fronds are approximately the same size in each test chamber, and the number of plants and fronds must be identical or as nearly identical as possible in each test chamber. (For example, three four-frond plants and one three-frond plant, for a total of 15 fronds, could be added to each test chamber.) A total of at least 12 but no more than 16 fronds is recommended.

11.4.3 The test begins when the plants are placed in each test chamber, which already contains test solution. The plants must be either:

11.4.3.1 Impartially assigned to the test chambers by placing one plant in each test chamber, and continuing the process until each chamber contains the desired number of plants and fronts, or

11.4.3.2 Assigned either by random assignment of one plant to each test chamber, random assignment of a second plant to each test chamber, etc., or by total randomization.

11.4.4 It might be convenient to assign plants to other containers, and then add them to the test chambers all at once.

11.5 *Duration of Test*—The test ends 7 days after plants are initially placed in test solutions containing test material. A shorter test duration might not be sufficient for toxicity to be demonstrated, whereas a longer test duration might allow the duckweed to adjust to the presence of the test material and permit extensive growth, increasing the difficulty in enumerating fronds.

11.6 *Biological Data*—Results of toxicity tests with *Lemna gibba* G3 should be calculated based on one or more measurements of the biomass in each test chamber. Because the results are calculated based upon growth in each treatment relative to that in the control, an initial measurement or estimate of biomass in each test chamber must be made. Indeed, the amount of duckweed initially placed in each test chamber must be identical or as nearly identical as possible. (Because growth occurs during the test, initial differences in biomass will be magnified and may obscure treatment-related effects.) A variety of methods may be used to measure or estimate biomass. The most common and simplest indirect measurement of biomass is determination of the number of plants or the number of fronds. In order to minimize subjective decisions on frond maturity, every frond that visibly projects beyond the edge of the parent frond should be counted as a separate frond. Fronds that have lost their pigmentation should not be counted. Frond number, plant number, root number, dry biomass and total root length are highly related to each other, but dry biomass (constant at 60°C) is the most objective and reproducible of the endpoints **(14)**. Root length **(10)**, fresh biomass **(10, 16)**, C-14 uptake **(17)**, chlorophyll **(17)**, and especially for axenic cultures **(16)** total Kjeldahl nitrogen and chlorophyll may also be measured to give additional information. Observations of change in color, break-up of plants, and destruction of roots should be included. All plants used in a test should be destroyed at the end of the test.

11.7 *Other Measurements*:

11.7.1 pH should be measured at the beginning and end of the test in the controls and in the high, medium, and low test concentrations. Precautions should be taken to avoid cross contamination.

11.7.2 Because test chambers are placed in a constant-temperature room, environmental chamber, or incubator, measurement of the air temperature at least hourly, or daily measurement of the maximum and minimum air temperature, may be made instead of any measurements in test chambers because the temperature of the air will probably fluctuate more than that of the test solutions. It is impractical to measure temperature in the test chambers when axenic conditions are to be maintained. Alternatively, one or two extra test chambers may be prepared for the purpose of measuring water temperature during the test.

11.7.3 It is desirable to determine the concentration of the test material in at least the control and the high, medium, and low concentrations of test material at the beginning and end of the test. If the concentrations are measured, results should be calculated based upon the initial concentrations and may also be calculated based on the average concentrations. Refer to Guides E 729 and E 1192 for information on the collection of samples of test solutions.

12. Analytical Methodology

12.1 If samples of growth medium, stock solutions, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(18)** to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

12.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist or are not sensitive enough, methods should be obtained from other reliable sources **(19)**.

12.3 The precision and bias of each analytical method used should be determined in the growth medium used. When appropriate, reagent blanks, recoveries, and standards should be included whenever samples are analyzed.

13. Acceptability of Test

13.1 A test should usually be considered unacceptable if one or more of the following occurred, except that if, for example, temperature was measured numerous times, one deviation of

more than 4°C (see 13.1.9) might be inconsequential. However, if temperature was measured only a minimal number of times, one deviation of more than 4°C might indicate that more deviations would have been found if temperature had been measured more often.

13.1.1 All test chambers and covers were not identical,

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

13.1.3 A required growth medium or solvent control was not included in the test or, if the concentration of solvent was not the same in all treatments that contained test material, the concentration of solvent in the range used affected growth of the test species,

13.1.4 The test organisms had not been cultured in growth medium and at the same temperature and light intensity as used in the test for at least the last eight weeks before the test,

13.1.5 The duckweed plants were not impartially or randomly assigned to the test chambers,

13.1.6 The test lasted less than 7 days,

13.1.7 Frond number in any control test chamber at the end of the test was not at least five times that at the beginning of the test,

13.1.8 Temperature was not measured as specified in 11.7.2,

13.1.9 The difference between the highest and lowest measured temperatures was greater than 4°C,

13.1.10 Any measured light intensity differed by more than 15 % from the selected light intensity, or

13.1.11 The number of plants was not the same and the number of fronds was not the same in each test chamber at the start of the test.

14. Calculation and Results

14.1 The results should be expressed in terms of the IC50. The NOEC may also be calculated. Both of these endpoints have utility and are acceptable measures of toxicity to aquatic plants **(11)**. It may be possible to determine both endpoints from a single data set.

14.2 To determine the IC50, calculate the percent inhibition (% *I*) for each test chamber in each treatment other than the control treatment(s). Percent inhibition is calculated as

$$\% I = 100(M - X)/M \quad (1)$$

where:

M = average increase in biomass in the control test chambers, and

X = increase in biomass in the test chamber.

(The increase in frond number, for example, is determined by subtracting the initial frond number from the final frond number.) The % *I* for each test chamber should be plotted against the corresponding concentration of test material after transformation of % *I* or concentration, or both, if appropriate. The IC50 can then be obtained from a line of best fit by determining the concentration corresponding to % $I = 50$. If % *I* is between 0 and 100 for fewer than two test chambers, only an approximate IC50 can be determined. Alternatively, if two or more test chambers gave % *I* between 0 and 100, appropriate linear or nonlinear regression techniques **(20)** can be used to calculate the IC50 and its 95 % confidence limits. A variety of regression models will usually give nearly the same IC50 from a set of data. However, only the correct model, which is not known to be available at this time, will appropriately take into account the number of test chambers per treatment, the range of concentrations tested, and the variance within each treatment, especially within the control treatment(s), and give the correct confidence limits.

14.2.1 Alternatively, the values for *X* may be plotted against the corresponding concentrations of test material, after transformation of *X* or concentration, or both, if appropriate, and the IC50 determined by graphical or statistical interpolation to the concentration of test material at which a line of best fit = $M/2$.

14.2.2 An IC near an extreme of toxicity, such as an IC5 or IC95, should not be calculated unless at least one concentration of test material caused a percentage inhibition in growth, other than 0 or 100 %, near the percentage for which the IC is to be calculated. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that caused only a small percentage, such as 5 %, inhibition in growth, or to report the lowest concentration of test material that actually caused a large percentage inhibition in growth. These alternatives are usually more reliable than reporting a calculated result such as an IC5 or IC95 unless several concentrations caused percent inhibitions close to 5 % or 95 %.

14.3 To determine the NOEC (no observed effect concentration), perform a hypothesis test to determine which of the tested concentrations of test material caused a statistically significant inhibition of growth. If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and tests of heterogeneity. Then a pairwise comparison technique, contingency table test, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of the results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test. The percent inhibition actually observed at the concentration considered the NOEC should be calculated.

15. Documentation

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

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

15.1.2 Source of the test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and identity and concentration(s) of any solvent used,

15.1.3 Description of the preparation of the growth medium,

15.1.4 Source of test species, scientific name and clone, name of person who identified the species and the taxonomic key used, method used to identify the clone, and culture procedures used,

15.1.5 Description of the experimental design, test chambers and covers, volume of solution in the chambers, and the number of plants and fronds per test chamber at the beginning of the test.

15.1.6 Average and range of the measured temperature and light intensity and the method of measurement or monitoring or both.

15.1.7 Methods used for, and results (with standard deviations or confidence limits) of, chemical analyses of concentration(s) of test material, including validation studies and reagent blanks,

15.1.8 Method(s) used for measuring or estimating biomass, for example, dry biomass or number of fronds,

15.1.9 A table of data on the biomass at the beginning and end of the test in each test chamber in each treatment, including the control(s), in sufficient detail to allow independent statistical analysis.

15.1.10 The IC50 (or other IC value), its 95 % confidence limits, and calculation method(s) used; the NOEC, the percent inhibition caused at this concentration, and calculation method(s) used; specify whether results are based on measured concentrations; for commercial products and formulations, specify whether results are based on active ingredient,

15.1.11 Any stimulation found in any treatment, and

15.1.12 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 results.

16. Keywords

16.1 aquatic plants; aquatic toxicity testing; duckweed; *Lemna gibba*

APPENDIX

(Nonmandatory Information)

X1. GROWTH MEDIA

X1.1 *Lemna gibba* G3 has been cultured and tested successfully in the media described in this appendix. Other media may also be used; however, it should be demonstrated that the medium supports an increase in biomass of at least five-fold within 7 days in the controls.

X1.2 Hoagland's E+ medium (see Table X1.1) has been historically used for culturing *Lemna gibba* G3 by botanists. This medium contains sucrose, yeast, and bacto-tryptone. In addition, the medium contains 9 mg/L EDTA and has a pH of 4.60. The characteristics of this medium make it undesirable for toxicity testing, as the addition of carbon sources and the low pH may complex and alter test materials, respectively.

TABLE X1.1 Preparation of Hoagland's E+ Medium (22, 23)

Solution	Substance	Concentration of Substance in Stock Solution, g/L	Amount in Growth Medium, mL/L[A,B]
A	$Ca(NO_3)\cdot 4H_2O$	59.00	20
	KNO_3	75.76	
	KH_2PO_4	34.00	
	6 mL 6N HCl		
B	Tartaric acid	3.00	1
C	$FeCl_3 6H_2O$	5.40	1
D	EDTA	9.00	1
	8 mL 6N KOH		
E	$MgSO_4\cdot 7H_2O$	50.00	10
F	H_3BO_3	2.86	1
	$ZnSO_4\cdot 7H_2O$	0.22	
	$Na_2MoO_4\cdot 2H_2O$	0.12	
	$CuSO_4\cdot 5H_2O$	0.08	
	$MnCl_2\cdot 4H_2O$	3.62	
G	Sucrose	...	10.00 g/L
H	Yeast extract	...	0.10 g/L
I	Bactotryptone	...	0.60 g/L

[A]Use reagent-grade chemicals. Make growth medium up to 1 L with glass distilled or deionized water. Adjust the pH to 4.60 with KOH or HCl. Autoclave 20 min at 121°C and 1.1 kg/cm^2.

[B]It has been shown **(14)** that growth of *Lemna gibba* G3 is enhanced by the addition of the following to the growth medium:

Se 4.2 µg/L
V 25.6 µg/L
Co 20.3 µg/L
Sn 457 µg/L

X1.3 Hoagland's medium without EDTA or sucrose (see Table X1.2) has been recommended by the U.S. Environmental Protection Agency for toxicity testing with *Lemna gibba* G3 **(21)**. This is the same as Hoagland's E + medium except the sucrose, bacto-tryptone, yeast, and EDTA are omitted. This medium was used by Hillman **(22)** in experiments with *Lemna gibba* G3. The pH of this medium is 5.0, which may not be desirable for use with many test materials.

X1.4 20X-AAP medium (see Table X1.3) is a modification of AAP medium, the medium used for toxicity testing with microalgae (see Guide E 1218). This medium contains the same nutrients as the AAP medium but at 20 times the

TABLE X1.2 Preparation of M-Hoagland's Medium Without Sucrose or EDTA (21)[A,B]

Chemical	Amount, mg
KH_2PO_4	680
KNO_3	1515
$Ca(NO_3)_2\cdot 4H_2O$	1180
$MgSO_4\cdot 7H_2O$	492
$FeCl_3\cdot 6H_2O$	5.40
Tartaric acid	3.00

[A]Use reagent grade chemicals. Add the chemicals in this table to distilled or deionized water (final volume to be 1 L).

[B]Add 1 mL of the micronutrient stock solution (solution F in Table X1.1) and bring the volume to 1 L. Autoclave for 20 min at 121°C and 1.1 kg/cm^2. Adjust the pH of the cooled medium to 5.0 ± 0.1 with 0.1 N KOH or HCl.

TABLE X1.3 Preparation of 20X-AAP Medium[A]

Macronutrients			
Stock Solutions		Nutrient Composition of Prepared Medium	
Compound	Concentration, g/L	Element	Nominal Concentration, mg/L
$NaNO_3$	25.500	N	84.00
$NaHCO_3$	15.000	Na	220.02
		C	42.86
K_2HPO_4	1.044	K	9.38
		P	3.72
$MgCl_4$ $7H_2O$	14.700	S	38.22
$MgCl_2$ $6H_2O$	12.164	Mg	58.08
$CaCl_2$ $2H_2O$	4.410	Ca	24.04
Micronutrients			
Stock Solution		Nutrient Composition of Prepared Medium	
Compound	Concentration, mg/L	Element	Nominal Concentration, µg/L
H_3BO_3	185.520	B	649.20
$MnCl_2$ $4H_2O$	415.610	Mn	2307.48
$ZnCl_2$	3.271	Zn	31.40
$CoCl_2$ $6H_2O$	1.428	Co	7.08
$CuCl_2$ $2H_2O$	0.012	Cu	0.08
Na_2MoO_4 $2H_2O$	7.260	Mo	57.56
$FeCl_3$ $6H_2O$	160.000	Fe	661.02
Na_2EDTA $2H_2O$	300.000	...	...

[A]Add 20 mL of each of the six macronutrient stock solutions and 20 mL of the micronutrient stock solution, in the order listed in this table, to approximately 800 mL of deionized or distilled water with mixing after each addition. Bring the volume to 1 L and adjust the pH to 7.5 ± 0.1 with 0.1 *N* sodium hydroxide or hydrochloric acid. Filter the medium through a 0.22-µm pore size membrane filter into a sterile container.

concentration. The pH of this medium is 7.5, it is entirely inorganic (except for the EDTA) and the ionic strength is much less than in the Hoagland's medium.

REFERENCES

(**1**) Hillman, W. S., and Culley, D. D., Jr., "The Use of Duckweed," *American Scientist*, Vol 66, 1978, pp. 442–451; Hillman, W. S., "The Lemnaceae, or Duckweed, a Review of the Descriptive and Experimental Literature," *Botanical Review*, Vol 27, 1961, pp. 221–287.

(**2**) Harvey, R. M., and Fox, J. L., "Nutrient Removal Using *Lemna minor*," *Journal of the Water Pollution Control Federation*, Vol 45, 1973, pp. 1928–1938; Culley, D. D., Jr. and Epps, E. A., "Use of Duckweed for Waste Treatment and Animal Feed," *Journal of the Water Pollution Control Federation*, Vol 45, 1973, pp. 337–347; O'Brien, W. J., "Use of Aquatic Macrophytes for Wastewater Treatment," *Environmental Engineering*, Vol 107, 1981, pp. 681–698; Porath, D., and Pollock, J., "Ammonia Stripping by Duckweed as Its Feasibility in Circulating Aquaculture," *Aquatic Botany*, Vol 13, 1982, pp. 125–131.

(**3**) Lewis, W. M., and Bender, M., "Effect of Cover of Duckweeds and the Algal *Pithophora* Upon the Dissolved Oxygen and Free Carbon Dioxide of Small Ponds," *Ecology*, Vol 42, 1961, pp. 602–603.

(**4**) U.S. Environmental Protection Agency, *Federal Register*, Vol 49, Feb. 7, 1984, pp. 4551–4554.

(**5**) For example, see: International Technical Information Institute, *Toxic and Hazardous Industrial Chemicals Safety Manual*, Tokyo, Japan, 1977; Sax, N. I., *Dangerous Properties of Industrial Materials*, 5th Ed., Van Nostrand Reinhold Co., New York, NY, 1979; Patty, R. A., ed., *Industrial Hygiene and Toxicology*, Vol II, 2nd Ed., Interscience, New York, NY, 1963; Hamilton, A., and Hardy, H. L., *Industrial Toxicology*, 3rd Ed., Publishing Sciences Group, Inc., Acton, MA, 1974; Gosselin, R. E., Hodge, H. C., Smith, R. P. and Gleason, M. N., *Clinical Toxicology of Commercial Products*, 4th Ed., Williams and Wilkins Co., Baltimore, MD, 1976.

(**6**) For example, see: Green, M. E., and Turk, A., *Safety in Working with Chemicals*, Macmillan, New York, NY, 1978; National Research Council, *Prudent Practices for Handling Hazardous Chemicals in Laboratories*, National Academy Press, Washington, DC, 1981; Walters, D. B., ed., *Safe Handling of Chemical Carcinogens, Mutagens, Teratogens, and Highly Toxic Substances*, Ann Arbor Science, Ann Arbor, MI, 1980; Fawcett, H. H., and Wood, W. S., eds., *Safety and Accident Prevention in Chemical Operations*, 2nd Ed., Wiley-Interscience, New York, NY, 1982.

(**7**) National Council on Radiation Protection and Measurement," Basic Radiation Protection Criteria," NCRP Report No. 39, Washington, DC, 1971; Shapiro, J., *Radiation Protection*, 2nd Ed., Harvard University Press, Cambridge, MA, 1981.

(**8**) National Institutes of Health, "NIH Guidelines for the Laboratory Use of Chemical Carcinogens," NIH Publication No. 81-2385, Bethesda, MD, 1981.

(**9**) Fekete, A., Riemer, D. N., and Motto, H. L., "A Bioassay Using Common Duckweed to Evaluate the Release of Available Phosphorus from Pond Sediments," *Journal of Aquatic Plant Management*, Vol 14, 1976, pp. 19–25; Nasu, Y., and Kugimoto, M., "Lemna (Duckweed) as an Indicator of Water Pollution. 1. The Sensitivity of *Lemna paucicostata* to Heavy Metals," *Archive of Environmental Contamination and Toxicology*, Vol 10, 1981, pp. 159–169; Hutchinson, T. C. and Czyrska, H., "Heavy Metal Toxicity and Synergism to Floating Aquatic Weeds," *Internationale Vereinigung fur Theoretische und Angewandte Limnologie*, Vol 19, 1975, pp. 2102–2111; Stanley, R. A., and Madewell, C. E., "Chemical Tolerance of *Lemna minor*L.," Circular Z-72, TVA, Muscle Shoals, AL; Wang, W., "Toxicity Tests of Aquatic Pollutants by Using Common Duckweed," *Environmental Pollution* (B), Vol 11, 1986, 1–14; Wang, W., "The Effect of River Water on Phytotoxicity of Barium, Cadmium, and Chromium Ions,"

Environmental Pollution (B), Vol II, 1986, pp. 193–204.

(**10**) Bishop, W. E., and Perry, R. L., "The Development and Evaluation of a Flow-Through Growth Inhibition Test with Duckweed (*Lemna minor*)," *ASTM STP 737*, ASTM, 1981, pp. 421–435.

(**11**) Hughes, J. S., Alexander, M. M., and Balu, K., "An Evaluation of Appropriate Expressions of Toxicity in Aquatic Plant Bioassays as Demonstrated by the Effects of Atrazine of Algae and Duckweed," *Aquatic Toxicology and Hazard Assessment: 10th Volume, ASTM STP 971*, W. J., Adams, G. A., Chapman, and W. G., Landis, eds., ASTM, Philadelphia, 1988, pp. 531–547.

(**12**) Veith, G. D., and Comstock, V. M., "Apparatus for Continuously Saturating Water with Hydrophobic Organic Chemicals," *Journal of the Fisheries Research Board of Canada*, Vol 32, 1975, pp. 1849–1951; Gingerich, W. H., Seim, W. K., and Schonbrod, R. D., "An Apparatus for the Continuous Generation of Stock Solutions of Hydrophic Chemicals," *Bulletin of Environmental Contamination and Toxicology*, Vol 23, 1979, pp. 685–689; Phipps, G. L., Holcombe, G. W., and Fiandt, J. T., "Saturator System for Generating Toxic Water Solutions for Aquatic Bioassays," *Progressive Fish-Culturist*, Vol 44, 1982, pp. 115–116.

(**13**) Correll, D. S., and Correll, H. B., *Aquatic and Wetland Plants of Southwestern United States*, Environmental Protection Agency, Washington, DC, 1972; Clark, H. L., and Thieret, J. W., "The Duckweeds of Minnesota," *The Michigan Botanist*, Vol 7, 1968, pp. 67–76; den Hartog, C., and van der Plas, F., "A Synopsis of the Lemnaceae," *Blumea*, Vol XVIII, 1970, pp. 355–368; Weik, K. L., and Mohlenbrock, R. H.," Contribution of a Flora of Illinois No. 3, Lemnaceae," *Transactions of the Illinois State Academy of Science*, Vol 61, 1968, pp. 382–399; McClure, J. W., and Alson, R. E., "A Chemotaxonomic Study of Lemnaceae," *American Journal of Botany*, Vol 53, 1966, pp. 849–860.

(**14**) Cowgill, U. M., and Milazzo, D. P., "The Culturing and Testing of Two Species of Duckweed," accepted for publication, *Aquatic Toxicology and Hazard Assessment*, 12th Symposium, ASTM, 1989.

(**15**) Cohen, J., *Statistical Power Analysis for the Behavioral Sciences*, Academic Press, New York, NY, 1977; Natrella, M. G., "The Relationship Between Confidence Intervals and Tests of Significance," *American Statistician*, Vol 14, 1960, pp. 20–22.

(**16**) Said, M. Z. M., Culley, D. D., Standifer, L. C., Epps, E. A., Myers, R. W., and Boney, S. A., "Effect of Harvest Rate, Waste Loading, and Stocking Density on the Yield of Duckweeds," *Proceedings of World Mariculture Society*, Vol 10, 1979, pp. 769–780; Culley, D. C., Rejmankova, E., Kuet, J., and Frye, J. B., "Production, Chemical Quality and Use of Duckweeds (Lemnaceae) in Aquaculture, Waste Management, and Animal Feeds," *Journal of World Mariculture Society*, Vol 12, 1981, pp. 27–49.

(**17**) Lockhart, W. L., Billect, B. N., de March, B. E. G., and Muir, D. C. G., "Uptake and Toxicity of Organic Compounds: Studies with an Aquatic Macrophyte, Duckweed (*Lemna minor*)," *ASTM STP 802*, ASTM, 1983, pp. 460–468.

(**18**) Berg, E. L., ed., "Handbook for Sampling and Sample Preservation of Water and Wastewater," EPA-600/4-82-029. National Technical Information Service, Springfield, VA, 1982.

(**19**) For example, see: U.S. Environmental Protection Agency, "Methods for Chemical Analysis of Water and Wastes," EPA-600/4-79/020, (revised March 1983), National Technical Information Service, Springfield, VA, 1983; U.S. Geological Survey, *National Handbook of Recommended Methods for Water-Data Acquisition*, U.S. Dept. of the Interior, Reston, VA, 1977; American Public Health Association, American Water Works Association, and Water Pollution Control Federation, *Standard Methods for the Examination of Water and Wastewater*, 16th Ed., Washington, DC, 1985.

(**20**) Draper, N. R., and Smith, H., *Applied Regression Analysis*, 2nd Ed., Wiley, New York, NY, 1981, pp. 65–84.

(**21**) Holst, R. W., and Ellwanger, T. C., *Pesticide Assessment Guidelines, Subdivision J Hazard Evaluation: Nontarget Plants*, EPA 540/9-82-020, Washington, DC, 1982.

(**22**) Hillman, W. S., "Experimental Control of Flowering in *Lemna*. III. A Relationship Between Medium Composition and the Opposite Photoperiodic Response of *L. perpusilla* 6746 and *L. gibba* G3," *American Journal of Botany*, Vol 48, 1961, pp. 413–419.

(**23**) Cleland, C. F., and Briggs, W. R., *Plant Physiology*, Vol 42, 1967, pp. 1538–1561.