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

This standard is issued under the fixed designation E1415; 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 E1192), 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).

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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:*[2]

[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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[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.

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E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
E1218 Guide for Conducting Static Toxicity Tests with Microalgae
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 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 E943, and Guides E729 and E1023. For an explanation of units and symbols, refer to Practice IEEE/ASTM SI 10 .

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**).[3] 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 E1023) 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

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

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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 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, and so forth, 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[4] chemicals to deionized or distilled water. Recommended growth media are given in Appendix X1.

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

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9. Test Material

9.1 *General*—The test material should be reagent-grade[4] 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,

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

9.2.3 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 greatest

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