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Standard Guide for Conducting Static Toxicity Tests with Microalgae[1,2]

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1. Scope

1.1 This guide covers procedures for obtaining laboratory data concerning the adverse effects of a test material added to growth medium on growth of certain species of freshwater and saltwater microalgae during a static exposure. These procedures will probably be useful for conducting short-term toxicity tests with other species of algae, although modifications might be necessary. Although the test duration is comparable to an acute toxicity test with aquatic animals, an algal toxicity test of short duration (72, 96 or 120 h) allows for examination of effects upon multiple generations of an algal population and thus should not be viewed as an acute toxicity test.

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 toxicity tests with microalgae.

1.3 These procedures are applicable to many 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 materials as aqueous effluents (see Guide E1192), leachates, oils, particulate matter, sediments, and surface waters. Static tests might not be applicable to materials that are highly volatile, are rapidly biologically or chemically transformed in aqueous solutions, or are removed from test solutions in substantial quantities by the test vessels or organisms during the test. (1)[3] However, practical flow-through test procedures with microalgae have not been developed.

1.4 Results of tests using microalgae should usually be reported in terms of the 96-h (or other time period) IC50 (see 3.2.5) based on reduction in 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.

1.5 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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[2] This standard guide is a document, developed using the consensus mechanisms of ASTM, that provides guidance for the selection of procedures to accomplish a specific test, but which does not stipulate specific procedures.

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

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[4]

D1129 Terminology Relating to Water

D3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters

D3978 Practice for Algal Growth Potential Testing with *Pseudokirchneriella subcapitata*

D4447 Guide for Disposal of Laboratory Chemicals and Samples

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System) (Withdrawn 1997)[5]

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

E1733 Guide for Use of Lighting in Laboratory Testing

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

3. Terminology

3.1 *Definitions:*

3.1.1 The words "must," "should," "may,"" can," and "might" have very specific meanings in this guide.

3.1.1.1 *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". Therefore the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can".

3.1.1.2 *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).

3.1.1.3 *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.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *algicidal*—having the property of killing algae.

3.2.2 *algistatic*—having the property of inhibiting algal growth.

3.2.3 *biomass*—the dry weight of living matter present in a population and expressed in terms of a given area or volume, for example, mg algae per liter. Because biomass is difficult to measure accurately, surrogate measures of biomass, such as cell counts, are typically used in this test.

3.2.4 *growth rate*—the increase in biomass per unit of time.

3.2.5 *IC50*—a statistically or graphically estimated concentration 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. Alternative values for inhibition, such as 10 % or 20 %, are referred to as IC10 or IC20.

3.2.6 *standing crop*—the algal biomass at the end of the test.

3.2.7 *yield*—the algal biomass at the end of the test minus the algal biomass at the beginning of the test.

3.3 For definitions of other terms used in this standard, refer to Guides E729 and E1023 and Terminology E943. For explanation of units and symbols, refer to Practice E380.

4. Summary of Guide

4.1 In each of two or more treatments, organisms of one species of microalgae are maintained in replicate test vessels using the static technique. The test duration is typically 96 h, but shorter periods (for example, 72 h) have been used for fast-growing algae and longer periods (for example, 120 h) may be necessary for slower-growing algae. In each of the one or more control treatments, the algae are maintained in growth medium to which no test material has been added in order to provide the following: a measure of the acceptability of the test by giving an indication of the quality of the algae 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 algae are maintained in growth medium to which test material has been added to achieve a selected concentration. Specified data on population growth are obtained during the test and are usually analyzed to determine the IC50 based on reduction in growth.

5. Significance and Use

5.1 Tests with algae provide information on the toxicity of test materials to an important component of the aquatic biota and might indicate whether additional testing (**2**) is desirable. Specific testing procedures under various regulatory jurisdictions follow procedures similar to those described in this Guide (**3**, **4**). Users should consult with any specific regulatory

[4] 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.

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

requirements to determine the applicability and consistency of this standard with such requirements.

5.2 Algae are ubiquitous in aquatic ecosystems, where they incorporate solar energy into biomass, produce oxygen, function in nutrient cycling and serve as food for animals. Because of their ecological importance, sensitivity to many toxicants, ready availability, ease of culture, and fast growth rates (rendering it possible to conduct a multi-generation test in a short period of time), algae are often used in toxicity testing.

5.3 Results of algal toxicity tests might be used to compare the sensitivities of different species of algae and the toxicities of different materials to algae and to study the effects of various environmental factors on results of such tests.

5.4 Results of algal toxicity tests might be an important consideration when assessing the hazards of materials to aquatic organisms (See Guide E1023) or deriving water quality criteria for aquatic organisms (**5**).

5.5 Results of algal toxicity tests might be useful for studying biological availability of, and structure-activity relationships between, test materials.

5.6 Results of algal toxicity tests will depend on the temperature, composition of the growth medium, and other factors. These tests are conducted in solutions that contain concentrations of salts, minerals, and nutrients that greatly exceed those in most surface waters. These conditions may over- or under-estimate the effects of the test material if discharged to surface waters.

6. Apparatus

6.1 *Facilities*—Cultures and test vessels should be maintained in rooms, incubators, or environmental chambers with constant temperatures (see 11.2) and appropriate illumination (see 11.3). A water bath is generally not acceptable because it prevents proper illumination of the test vessels. 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, algae should not be cultured in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned.

6.2 *Equipment*—Some or all of the following will be needed:

6.2.1 *Centrifuge,*

6.2.2 *Centrifuge Tubes,* glass or polycarbonate with screw-cap lids,

6.2.3 *Rotary or Oscillation Shaker,* with variable speed control capable of 100 r/min (or oscillations per minute),

6.2.4 *Erlenmeyer Flasks,* borosilicate glass, or polycarbonate

6.2.5 *Stainless Steel Caps, Shimatsu Enclosures, Foam Plugs, Glass Caps,* or *Standard Screw Caps*, (plastic/bakelite) (all closures should be loose-fitting),

6.2.6 *Pipets,* Eppendorf or equivalent,

6.2.7 *Filtration Apparatus,*

6.2.8 *Membrane Filters,* with 0.45 µm and 0.22 µm pore size,

6.2.9 *Analytical Balance,*

6.2.10 *Autoclave or Microwave Oven,*

6.2.11 *pH Meter,*

6.2.12 *Calibrated Light Meter,* reading in µmol $m^{-2}s^{-1}$ or lumens,

6.2.13 *Microscope,* capable of 100× to 400× magnification,

6.2.14 *Hemacytometer Counting Chamber* or *Plankton Counting Chamber* and *Ocular Micrometer*,

6.2.15 *Particle Counter,* with 70 µm or 100 µm aperture tube, and (optional) mean cell volume computer, or

6.2.16 *Fluorometer,* equipped to measure chlorophyll *a*, or

6.2.17 *Spectrophotometer,* to measure cell densities in log phase cultures.

6.3 *Test Vessels:*

6.3.1 In a toxicity test with aquatic organisms, test chambers (also referred to as test vessels) are defined as the smallest physical units between which there are no water connections. Vessels should be covered to keep out extraneous contaminants, especially bacteria and undesirable algae. Because algae consume carbon dioxide, the covers used for algal tests must not prevent the passage of air. All vessels and covers in a test must be identical.

6.3.2 Sterile Erlenmeyer flasks of borosilicate glass or polycarbonate are usually used as test and culture vessels. Any size flask can be used as long as the test solution volume does not exceed 50 % of the flask volume for tests conducted on a shaker, and not more than 20 % of the flask volume for tests not conducted on a shaker. The proper solution/volume ratio should be determined for each test species in each laboratory because the ratio is dependent on the species and conditions.

6.4 *Cleaning:*

6.4.1 Test vessels 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.) At the end of the test, all items that are to be used again should be immediately emptied, rinsed with water, cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals), cleaned with a non-phosphate detergent using a stiff bristle brush to loosen any attached materials and rinsed at least twice with deionized or distilled water. Acid is often used to remove mineral deposits.

6.4.2 If an electronic particle counter is to be used to count algal cells, the final rinse should be with water that has been filtered through a 0.22-µm membrane filter.

6.4.3 Test vessels may be dried in an oven at 50 °C to 100 °C and capped with either stainless steel, foam or glass caps, or Shimatsu closures. Glassware should be sterilized by autoclaving for 20 min at 121°C and 1.1 kg/cm^2 or by microwaving (**6**). Hand-made cotton plugs should not be used. The acceptability of foam plugs should be investigated prior to use because some brands have been reported to be toxic.

6.5 *Acceptability*—Before a toxicity test is conducted with algae in new test facilities, it is desirable to conduct a "non-toxicant" test, in which all test vessels contain growth medium with no added test material, to determine before the first test whether algae 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 magnitude of the between vessel variance.

7. Hazards

7.1 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 vessels and ventilating the area surrounding the vessels, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**7**), recommended handling procedures (**8**), and chemical and physical properties of the test material, as available on the material safety data sheet, should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (**9**) and with test materials that are, or are suspected of being, carcinogenic (**10**).

7.2 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.

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 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.

8. Growth Medium

8.1 Growth medium for tests with freshwater algae is prepared by adding appropriate amounts of specified chemicals (see Annex A1) to ASTM Type I water. Alternatively, depending upon the purpose of the toxicity test, a natural water can be used as the basis for preparing the medium.

8.2 Growth medium water for tests with saltwater algae is prepared by adding appropriate amounts of specified chemicals to natural or artificial saltwater (see Annex A2; "Enriched" medium) or to ASTM Type I water (see Annex A3; "Complete" medium). A variety of salt waters are acceptable, for example, various reconstituted waters prepared with salts[6] or filtered (0.22-µm membrane filter) natural seawater. Salinity should be between 24 and 35 g/kg. Salinity may be adjusted by adding ASTM Type I water, NaCl, or sea salts.

8.3 If using a natural water to prepare the medium, indigenous plankton should be removed from the water before use especially if the test is used to determine the quality of a surface water. Two methods are available. First, the water can be filtered through a 0.22 µm membrane filter. Second, the water can be filtered through a 0.45 µm membrane filter and then for saltwater algae the water should be heated for 4 h at 60 °C, and for freshwater algae, the water should be sterilized by microwaving or autoclaving at 1.1 kg/cm^2 and 121 °C for 20 min. (Autoclaving might cause the formation of a precipitate.) Filtration through a 0.22 µm membrane filter will remove indigenous organisms. After filtration, microwaving, or autoclaving, the water should be equilibrated by letting the water sit in a loosely capped vessel or aerating with sterile air for 1 to 2 h. Air used for aeration should be sterile (filtered through a 0.22 µm bacterial filter) and free of fumes, oil and water; filters to remove oil and water are desirable.

8.4 It may be desirable to reduce the amount or omit EDTA from the medium in toxicity testing if it is suspected that the chelator will interact with the test material. However, the amount of EDTA in the freshwater medium is minimal and is necessary to obtain consistent and acceptable algal growth. If EDTA is reduced or omitted from the freshwater medium, two sets of controls should be used, one of medium with the recommended concentration of EDTA and one of medium with the reduced concentration or without EDTA. These controls should be evaluated according to the discussion in 9.2.4.3 and 9.2.4.4

9. Test Material

9.1 *General*—The test material should be reagent-grade[7] 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, and volatility in the growth medium.

9.1.3 Measured or estimated toxicity to the test species or a similar species. (If nothing is known about the toxicity to the test species, a range-finding test is suggested).

[6] Salts such as Instant Ocean, available from Aquarium Systems, 8141 Tyler Blvd., Mentor, OH have been found suitable for this purpose.

[7] *Reagent Chemicals, American Chemical Society Specifications* . American Chemical Society, 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, U.K., and the *United States Pharmacopeial and National Formulary*. U.S. Pharamceutical Convention, Inc. (USPC), Rockville, MD.

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

9.1.5 Estimate of toxicity to humans.

9.1.6 Recommended handling procedures (see 7.1).

9.2 *Stock Solution:*

9.2.1 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 the growth medium. If a stock solution is used, 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. If the test material is subject to hydrolysis, preservation by adjustment of pH may be necessary, and, in this instance, it may be desirable to investigate the influence of any pH adjustments on the medium or the test organism.

9.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is growth medium. ASTM Type I 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 (**11**). The minimum necessary amount of a strong acid or base, or both, 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 less than the use of the minimum necessary amount of a strong acid or base.

9.2.3 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 the test species. Because of its low volatility and high ability to dissolve many organic chemicals, N,N-dimethylformamide is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as ethanol and acetone may also be used, but they might stimulate undesirable growth of microorganisms, and acetone is also quite volatile. If an organic solvent is used, it should be reagent-grade[7] or better and its concentration in any test solution should not exceed 0.5 mL⁄L, and preferably 0.1 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 ingredients of a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution.)

9.2.4 If a solvent other than growth medium 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 a solvent control is not required, a growth medium control must be included in the test.

9.2.4.1 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.

9.2.4.2 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 solvent test must be conducted to determine whether growth of the test species is related to the concentration of solvent over the range used in the toxicity test, or such a solvent test must have already been conducted using the same growth medium and test species. If growth is found to be related to the concentration of solvent, an algal test with that species 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, an algal test with that species in that medium may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

9.2.4.3 If the test contains both a growth medium control and a solvent control, the growth in the two controls should be compared using a t-test. 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.

9.2.4.4 If a statistical difference in growth is detected between the two controls, only the solvent control may be used for meeting the requirements of 13.1.7 and as the basis for calculation of results. If a t-test indicates that the solvent control and the control are different and if the concentration of solvent at each test material concentration is not equal, the use of hypothesis testing statistics for comparison of treatments to each other or to the solvent control is not appropriate. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of 13.1.7 and as the basis for calculation of results.(**12**)

9.2.5 If an organic solvent is used to prepare a stock solution, it might be desirable to conduct simultaneous tests 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 Depending on the nature of the test material, test solutions are prepared by one of two methods. In the first method, technical materials (solids or liquids) are tested by weight/volume concentration. The material may be added directly by weight to the growth medium or a stock solution may be prepared (see 9.2) and aliquots added to each test solution or test vessel. If it is not possible to prepare a homogeneous solution of the test material, it must be added directly to each test solution or test vessel. In the second method, aqueous effluents are tested by percent volume (volume/volume). Nutrients are added in the same quantities as in the medium (see Annex A1 – Annex A3) to 1 L of the effluent. This mixture, without sterilization by filtration or any

other alteration, is used as the 100 % test material concentration. The other test solutions are prepared on a volume-percent basis by diluting the 100 % effluent solution with growth medium. If the effluent itself contained nutrient(s), the treatments that contain test material will contain more of the nutrient(s) than will the control treatment. However, in most cases this is not critical for short-term tests. USEPA 2002, **(3)** describes a method for testing the effects of effluents on algae.

9.3.2 If the test is intended to allow calculation of the IC50, the test concentrations (see 11.1.1.1) should bracket the predicted IC50. The prediction might be based on the results of a test on the same or a similar test material with the same or a similar algal 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 ten. The greater the similarity between the range-finding test and the actual test, the more useful the range-finding test will be.

9.3.2.1 Testing materials at levels above their water solubility presents several difficulties. At test material loadings above the solubility (note a true concentration cannot exist above solubility and a term such as "loading" is used), test materials exist in a variety of aggregate forms (for example, particulates, crystals, liquid crystals, etc.) Relatively little is known about the uptake of aggregated compounds into biological membranes and the expression of toxicity as a result. In fact, toxicity may be due to certain physical effects (reduction in light penetration and interference with nutrient uptake by test material particulates, or flocculation of algae onto test material particles). For materials tested at loadings in excess of solubility, the use of data in risk assessments or for comparison with other test materials, is complicated by the lack of knowledge as to whether the effect is due to a physical effect or true toxicity. These difficulties suggest that toxicity testing at loadings above solubility should be discouraged. To ensure that solubility has been achieved in the toxicity test, it may be appropriate to test up to approximately twice the solubility limit in the medium being used. However, in this case toxicity should be expressed as the solubility limit (that is, no effect at the solubility limit, or 20% reduction in cell numbers at the solubility limit). Analytical verification of the solubility under the test conditions can be critical for test materials of limited aqueous solubility.

9.3.3 In some (usually regulatory) situations, it is only necessary to determine whether a specific concentration of test material 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 concentration (see 11.1.1.2), and it is not necessary to determine the IC50.

10. Test Organisms

10.1 *Species*—The suggested test species were selected because they are readily available, easy to culture in the laboratory, and have been successfully used; however, these species might not be the most sensitive. Their use is encouraged to increase comparability of results.

10.1.1 *Fresh Water*—The species most widely used for testing is the green alga *Raphidocelis subcapitata* formerly known as*Pseudokirchneriella subcapitataSelenastrum capricornutum* Printz [8] and the culture and test procedures described here are most applicable to it **(13)**. Other species that have been successfully used include *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*) Chodat and *Chlorella vulgaris* Beijerinck. Freshwater algae from other phyla can be used, such as the diatom, *Navicula pelliculosa* Brebisson Hilse. Other organisms that have been used in this test that were formerly classified as blue-green algae are now considered cyanobacteria, including *Microcystis aeruginosa* Kutzing and *Anabaena flos-aquae* (Lyngb.) De Brebisson.

10.1.2 *Salt Water*—The species most widely used for testing is the diatom, *Skeletonema costatum*, (Greville) Clevel., **(14)** also known as the newly classified species *Skeletonema marinoi***(15)**. Other species that have been successfully used are the centric diatom, *Thalassiosira pseudonana*, Hasle and Heimdal, the flagellate, *Dunaliella tertiolecta*, Butcher **(16)** and the goldenbrown alga, *Phaeodactylum tricornutum*.

10.1.3 Because the sensitivities of algal species often differ substantially, it is usually desirable to conduct tests with two or more species from different phyla. Different strains of the same species may differ also in sensitivity.

10.2 *Source*—Many species can be obtained from the American Type Culture Collection, the University of Texas Collection, the Bigelow Laboratory for Ocean Sciencesand Carolina Biological Supply.[9]

10.3 *Culture*—Key references for culturing algae are those of Stein and Provasoli **(17)**. Aseptic stock transfer should be performed weekly to maintain a continuous supply of cells in or near logarithmic growth phase. The volume transferred is not critical, but enough cells should be transferred to ensure a minimum visual lag period in growth (for example, 1.0 mL culture added to 50 mL medium in a 125 mL flask). Extreme care should be exercised to avoid contamination of stock cultures. If tests are conducted infrequently, long-term maintenance of the test species using a solid medium containing 1 % agar in sterile Petri plates or test tubes might be desirable.

10.4 *Quality*—A culture should not be used for starting a test if it is not in log growth phase, if visual examination at 400× shows it is contaminated by fungi or other algae, or if the health of the culture is doubtful in any respect. In order to assess culture health, sufficient experience with the test species should be developed prior to use in testing. When a testing facility receives a culture of a species that has not been

[8] Renamed by Gunnar Nygaard, Jirf Komárek, Jørgen Kristiansen and Olav M. Skulberg, 1986. Taxonomic designations of the bioassay alga NIVA-CHL1 ("Selenastrum capricornutum") and some related strains. Opera Botanica 90:5-46.

[9] Algal species are available from American Type Culture Collection 12301 Parklawn Dr., Rockville, MD 20852, from the University of Texas Algal Collection, Botany Department, Austin, TX 78712, from the Bigelow Laboratory for Ocean Sciences , National Center for Marine Algae and Microbiota, 60 Bigelow Drive, East Boothbay, Maine 04544, and from Carolina Biological Supply Company, 2700 York Road, Burlington, NC, 27215.

previously maintained in that facility, the species should be cultured over a period of at least six weeks to establish the ability to successfully maintain a healthy, reproducibly-growing culture. Once experience has been obtained with a particular species from a particular source, subsequent cultures should be maintained for at least two weeks after receipt prior to use in testing.

11. Procedure

11.1 *Experimental Design:*

11.1.1 For detailed guidance of experimental design and statistical analyses, refer to Practice E1847. Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and number of test vessels per treatment, should be based on the purpose of the test and the type of procedure that is to be used to calculate results (see Section 14). One of the following two types of experimental design will probably be appropriate in most cases.

11.1.1.1 An algal 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 growth medium or solvent control(s), or both, (see 9.2.3), algae are 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 uncertain, 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 9.3.3), only that concentration and the control(s) are necessary. Two additional concentrations at about one half and two times the specified concentration 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 reduced 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, that is defined as the smallest physical entity to which treatments can be independently assigned. Thus, the test vessel is the experimental unit. As the number of test vessels (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 significance test increases. With respect to factors that might affect results within test vessels and, therefore, the results of the test, all vessels in the test should be treated as similarly as possible. For example, the temperature in all test vessels should be as similar as possible unless the purpose of the test is to study the effect of temperature. Test vessels are usually arranged in one or more rows. Treatments must be randomly assigned to individual test vessel locations and may be randomly reassigned during testing. A randomized block design (with each treatment being present in each block, which may be a row or a rectangle) is preferable to a completely randomized design.

11.1.3 The minimum desirable number of test vessels per treatment should be calculated from the expected variance between test vessels within a treatment, and either the maximum acceptable confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing **(18)**. If such calculations are not made, at least three test vessels 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, proportionately fewer test vessels may be used for each treatment that contains test material, but not for the control treatment(s). Replicate test vessels (that is, experimental units) within a treatment are necessary in order to allow estimation of experimental error **(19)**. Because of the importance of the controls, it might be desirable to use more test vessels for the control treatment(s) than for each of the other treatments, such that if a control replicate is lost, sufficient replicates remain for statistical analyses.

11.2 *Temperature*—Tests with the recommended freshwater microalgae should be conducted at 24 ± 2°C and tests with the recommended saltwater microalgae should be conducted at 20 ± 2°C.

11.3 *Illumination*—Continuous "cool-white" fluorescent lighting should be used to provide 60 µmol $m^{-2}s^{-1}$ (4300 lm/m^2) for the recommended freshwater diatoms and green algae and 30 µmol $m^{-2}s^{-1}$ (2150 lm/m^2) for the recommended freshwater cyanobacteria. *For Thalassiosira*, 80 µmol $m^{-2}s^{-1}$ to 90 µmol $m^{-2}s^{-1}$ (5900 lm/m^2 to 6500 lm/m^2) is recommended and 60 µmol $m^{-2}s^{-1}$ (4300 lm/m^2) for *Skeletonema* **(14)**. The light fluence rate at each test vessel position should be measured and should not differ by more than 15% from the selected fluence rate. For further information on the use of lighting in laboratory testing, especially with regard to using other spectral regions (such as ultraviolet radiation), see Guide E1733. A photoperiod should be used with *Skeletonema* (that is, 14 h light: 10 h dark, or 16 h light: 8 h dark).

11.4 *Beginning the Test:*

11.4.1 A large enough batch of the growth medium should be prepared so that the desired volume can be placed in each control test vessel, the necessary volume of each test solution can be prepared, and the desired analyses can be performed (see 11.8). Enough test solution should be prepared for each treatment so that the desired volume can be placed in each test vessel and any desired analyses of water quality, test material, etc. (see 11.8) can be performed.

11.4.2 Each test vessel should be inoculated at an initial population density to allow sufficient growth under the test conditions without resulting in nutrient or carbon dioxide limitation. Recommended concentrations are as follows: