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Standard Guide for Conducting Static 96-h 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 96-h 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, dissolved oxygen, and pH and on such materials as aqueous effluents (see also Guide E 1192), leachates, oils, particulate matter, sediments, and surface waters. 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 solutions, or are removed from test solutions in substantial quantities by the test vessels or organisms during the test. 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 3.2.45) 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 E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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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.

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 determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents [3]

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water

D 3731 Practice for Measurement of Chlorophyll Content of Algae in Surface Waters

D 3978 Practice for Algal Growth Potential Testing with ~~*Selenastrum Capricornutum*~~*Pseudokirchneriella subcapitata*

D 4447 Guide for the Disposal of Laboratory Chemicals and Samples

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

E 729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E 943 Terminology Relating to Biological Effects and Environmental Fate

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E 1733 Guide for the Use of Lighting in Laboratory Testing

E 1847 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 propeerty 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.

~~3.2.4 *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.~~

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] 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*, ~~Vol 11.01.~~ volume information, refer to the standard’s Document Summary page on the ASTM website.

3.2.6 *standing crop*—the algal biomass ~~(dry weight, mg/L or cell concentration, cells/mL)~~ 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 E 729 and E 1023 and Terminology E 943. For explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 In each of two or more treatments, organisms of one species of microalgae are maintained ~~for 96 h~~ 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 ~~(increase in biomass)~~ are obtained during the test and are usually analyzed to determine the IC50 based on reduction in growth.

5. Significance and Use

~~5.1 Ninety-six hour tests~~

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 **(1)** [4] is desirable.

5.2 Al~~ga~~ae 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), al~~ga~~ae 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 E 1023) or deriving water quality criteria for aquatic organisms **(2)**.

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 ~~to exceed~~ 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 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,

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[4] The boldface numbers given in parentheses refer to a list of ~~ASTM Standards, Vol 11.05.~~ references at the end of the text.

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

6.2.15 *Particle Counter*, with 70- 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 ~~V~~Test vessels may be dried in an oven at 50 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 (**7**). 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 **(3)**, recommended handling procedures **(4)**, 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 **(5)** and with test materials that are, or are suspected of being, carcinogenic **(6)**.

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 ~~A1.1)~~ Annex A1) to ~~filtered deionized or distilled~~ 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 ~~deionized or distilled~~ ASTM Type I water (see Annex A3;

"Complete" medium). A variety of salt waters are acceptable, for example, various reconstituted waters prepared with salts[5] or filtered (0.22-µm membrane filter) natural seawater. Salinity should be between 24 and 35 g/kg. Salinity may be adjusted by adding ~~deionized or distilled~~ 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[6] 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).

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. ~~Distilled or deionized~~ 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 **(8)**. 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[6] 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.)

~~*Annual Book of ASTM Standards*, Vol 11.04.~~

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

[6] ~~*Annual Book*~~*Reagent Chemicals, American Chemical Society Specifications* . American Chemical Society, Washington, DC. For suggestions on the testing of ~~ASTM~~ reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, ~~Vol 14.02.~~ BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeial and National Formulary*. U.S. Pharamceutical Convention, Inc. (USPC), Rockville, MD.

9.2.4 If a solvent othen 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. ~~Adjustments for vessel-to-vessel heterogeneity might be necessary if more than one vessel is used. The use~~ 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.

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. ~~Weber et al.~~ USEPA 2002, **(9)** describes a method for testing the effects of effluents on algae.

9.3.2 If the test is intended to allow calculation of the ~~96-h~~ 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 actually 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 *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* Printz and ~~Printz (~~*Raphidocelis subcapitata*)[7] and the culture and test procedures described here are most applicable to it **(10)**. Other species that have been successfully used include ~~*Scenedesmus*~~*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, and the diatom, *Navicula pelliculosa*.~~ Brebisson.

10.1.2 *Salt Water*—The species most widely used for testing is the diatom, *Skeletonema costatum*, (Greville) Clevel., **(11)**. Other species that have been successfully used are the centric diatom, *Thalassiosira pseudonana*, Hasle and Heimdal, the flagellate, *Dunaliella tertiolecta*, Butcher **(12)** 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 and Carolina Biological Supply.[8]

10.3 *Culture*—Key references for culturing algae are those of Stein and Provasoli **(13)** . 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 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 E 1847. 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.

~~[7] The boldface numbers given in parentheses refer to a list~~

[7] Renamed by Gunnar Nygaard, Jirf Komárek, Jørgen Kristiansen and Olav M. Skulberg, 1986. Taxonomic designations of ~~references at~~ the bioassay alga NIVA-CHL1 ("Selenastrum capricornutum") and sofme relat~~h~~ed strains. Ope~~x~~ra Botanica 90:5-46.

~~[8] Salts such as Instant Ocean,~~

[8] Algal species are available from ~~Aquarium Systems, 8141 Tyler Blvd., Mentor, OH have been found suitable for this purpose.~~ American Type Culture Collection 12301 Parklawn Dr., Rockville, MD 20852, from the University of Texas Algal Collection, Botany Department, Austin, TX 78712, and from Carolina Biological Supply Company, 2700 York Road, Burlington, NC, 27215.

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 **(14)**. 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 **(15)**. Because of the importance of the ~~controls in the calculation of results,~~ 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 ~~blue-green algae.~~ cyanobacteria. *For Thalassiosira*, 80 to 90 µmol $m^{-2}s^{-1}$ (5900 to 6500 lm/m^2) is recommended and 60 µmol $m^{-2}s^{-1}$ (4300 lm/m^2) for *Skeletonema* (**11**). 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 E 1733. 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 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:

~~*Selenastrum capricornutum* and other freshwater green algae~~	~~1-2 × 10^4 cells/mL~~
Pseudokirchneriella subcapitata and other freshwater green algae	1-2 × 10^4 cells/mL
Navicula pelliculosa	1-2 × 10^4 cells/mL
Microcystis aeruginosa	5 × 10^4 cells/mL
Anabaena flos-aquae	1-2 × 10^4 cells/mL
Saltwater Species	1-2 × 10^4 cells/mL

The primary criterion for an inoculum concentration is that accurate biomass estimates can be obtained with the chosen method of measurement during the test, if necessary, and at the end of the test. Lower concentrations may be practical for some laboratories. Higher concentrations might be desirable if there are indications that logarithmic growth will not be attained within 4 days or if results are to be based on measurements made during the test. However, the upper limit of the inoculum should be no more than 10^5 cells/mL. After determining the concentration of algal cells in a logarithmically-growing stock culture, the following equation can be used to calculate the total volume of culture required for inoculation of all test vessels:

$$A = (B \times C \times D \times E)/F \quad (1)$$

where:

A = volume of stock culture required to prepare the inoculum, mL,
B = desired initial concentration of cells in test vessel, cells/mL,
C = volume of solution in one test chamber, mL,

D = number of test vessels,
E = factor of 1.2 to provide a surplus of inoculum, and
F = concentration of cells in the stock culture, cells/mL.

11.5 *Gas Exchange*—Gas exchange through closures is facilitated by continuous shaking at 100 ± 10 oscillations per minute or periodic hand shaking. The rate of oscillation should be determined at the start of the test. Closures must be loose-fitting or porous. Because continuous shaking of certain diatoms (that is, *Skeletonema*) may cause clumping or retard growth, they should be hand shaken once or twice daily. (It has been reported by at least one laboratory, however, that continuous shaking of *S. costatum* will result in acceptable growth). *Thalassiosira* should be hand shaken once a day.

11.6 *Duration of Test*—The test must last ~~96 hours (approximately) unless it has been shown that~~ at ~~some shorter duration the biomass in the control(s) is at~~ least ~~90 % of that at 96~~ 72 h. ~~Biomass~~ Population increase will reflect time passed, algal clone, incubation conditions, and medium employed. If desired, an optional recovery phase may follow the ~~96-hour~~ exposure phase (see 11.8.5). A test duration of 72 h is usually sufficient for freshwater green algae, while other algae may require 96 h or longer to demonstrate sufficient growth to allow reliable biomass measurements.

11.7 *Biological Data*—Results of algal toxicity tests should be calculated based on one or more measurements of biomass in each test vessel. A variety of methods can be used to measure or estimate biomass.

11.7.1 Dry weight actually measures ~~biomass, whereas the other techniques only estimate biomass. However,~~ biomass; however, dry weight is difficult to measure ~~accurately. In addition, an estimate of biomass that correlates well with the dry weight of a particular species under some conditions might not correlate well when the growth of that same species of algae is affected by a test material. Because test materials can affect algae in different ways, if dry weight is not measured, it may be desirable to estimate biomass using two different techniques. Cell counts and chlorophyll *a* are the more commonly used measurements.~~ accurately.

11.7.2 *Cell Counts*—Microscopic counting of cells can be performed using a hemocytometer or an inverted microscope with settling chambers **(16)**. Precision is proportional to the square root of the number of the cells counted. The precision decreases substantially when cell densities are less than 1×10^2 cells/mL. For microscopic counting, two samples should be taken from each flask and two counts made of each sample. Whenever feasible, at least 400 cells per flask should be counted in order to obtain ± 10 % accuracy at the 95 % confidence level. Counting is relatively time-consuming, but less so than determining dry weight.

11.7.2.1 An alternative method that is able to enumerate large numbers of cells very rapidly is to use an electronic particle counter **(10)**. If a particle counter is used, representative samples of cells should be also microscopically examined for morphological abnormalities at test termination. Automated particle counting, although the most rapid and sensitive method, has limitations **(17)**, some related to particle interferences. If the test solution does not have a low background in the particle size range of the test species, masking errors will result. ~~A fourth~~ An extra test vessel at each concentration containing test material and growth medium, but no algae, will allow measurement of particle interferences. The other methods are not affected by particulate material in the growth medium.

11.7.2.2 Cell chains of filamentous species, such as *Anabaena flos-aquae*, must be broken up prior to counting, regardless of the counting method selected. An effective method for reducing the length of the filaments without rupturing the cells is sonication. Subsamples from the test vessels should be sonicated for a sufficient duration to reduce the filaments to a length that is consistent between samples. The total volume of subsamples that will be required should be determined before the start of the test to allow sufficient test solution volume to avoid significant changes in the liquid surface-to-volume ratio, which can affect growth.

11.7.3 *Chlorophyll a*—This pigment can be measured spectrophotometrically *in vitro* or fluorometrically *in vivo* and *in vitro* **(16)**. Both methods are fairly rapid, but the spectrophotometric method lacks sensitivity at low cell densities, whereas the *in vivo* fluorometric method is very sensitive and requires only a very small sample volume. Relative fluorescence readings can be used to estimate relative biomass or a more definitive estimate can be made using chlorophyll *a* content (see Practice D 3731). If only a fluorometer is used, representative samples of cells also should be microscopically examined for morphological abnormalities at test termination.

11.7.4 Other measurements that have been used to estimate biomass include adenosinetriphosphate (ATP) **(18)** , carbon 14 assimilation **(19)**, optical density, and deoxyribonucleic acid (DNA) content **(20)**.

11.7.5 Regardless of which method(s) is used to measure biomass, the number of times that measurements must be made on each test vessel depends on the method used to calculate the ~~IC50~~ test endpoints (see Section 14). If the IC50 (or other test endpoints) are to be calculated based on ~~standing crop,~~ yield or average growth rate, biomass must be measured or estimated in each test vessel only at the beginning and end of the ~~test (see 11.6).~~ test. However, ~~if an IC50~~ it is ~~to be calculated based on either growth rate or area under the curve, or both, the~~ strongly recommended that biomass mueasurements be ~~measured at least~~ performed daily ~~during the test~~ in each test vessel to allow evaluation of test material effects during the test.

11.8 *Other Measurements*:

11.8.1 The pH in the control and the high, medium, and low test concentrations should be measured at the beginning and end of each test.

11.8.2 Because test vessels 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 any test vessels because the temperature of the air will probably fluctuate more than the temperature of the

test solutions. It is impractical to measure temperature in the test vessels when axenic conditions are to be maintained. Alternatively, one or two extra test vessels may be prepared for the purpose of measuring water temperature during the test.

11.8.3 Prior to the start of the test, the light fluence rate should be measured at each test vessel position. Light fluence rate should not deviate by more than 15% from the selected level. Layers of cheesecloth can be used to shade flask positions if necessary. Random repositioning of the test vessels during the test may be useful to minimize spatial differences.

11.8.4 Measurements of the concentration of the test material in the test solutions at the beginning and end of the test are desirable. Measurements before and after centrifugation or filtration can determine what percentage of the test material is not associated with the algae. If the concentrations are measured, results should be calculated based upon the initial concentrations and may also be calculated based upon the average concentrations. Refer to Guides E 729 and E 1192 for information on the collection of samples of test solutions.

11.8.5 *Determination of Algistatic and Algicidal Effects*—As defined by Payne and Hall (**1**), the algistatic concentration is the concentration that allows no net growth of the population of test organisms during exposure to the test material but permits regrowth when the organisms are placed in test material-free medium; the algicidal concentration is the lowest concentration tested which allows no net increase in population density during either the recovery or exposure period, that is, the organisms do not recover when transferred to test material-free medium. If it is desirable to distinguish algistatic from algicidal effects, a recovery phase may be initiated at the end of the ~~96-h~~ exposure period. The lowest test concentration with ~~96-h~~ final cell counts similar to or less than the initial inoculum level is subjected to the recovery phase. (If algal growth was not completely inhibited, the highest test concentration causing inhibition of algal growth is used). An aliquot of 0.5 mL from each of the replicate flasks of the selected test concentration is removed and combined in a single clean flask containing sufficient fresh growth medium to dilute the test material to an insignificant concentration. Aliquots from the control flasks are also transferred to clean medium. These flasks are incubated under the same conditions described for the exposure phase for a period of up to nine days. Biomass measurements are performed periodically during the recovery phase. The recovery phase may be discontinued as soon as growth has occurred.

12. Analytical Methodology

12.1 If samples of stock solution or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(21)** 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 **(22)** .

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 specimens are analyzed. If test solutions will be centrifuged or filtered to remove algae prior to analysis, the method validation should include an assessment of these procedures upon recovery.

13. Acceptability of Test

13.1 Usually consider unacceptable a ~~96-h~~ test with microalgae if one or more of the following occurred:

13.1.1 All test vessels and covers were not identical,

13.1.2 Treatments were not randomly assigned to test vessels,

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, or the concentration of solvent in the range used affected growth of the test species,

13.1.4 There were fewer than three replicates per treatment, except in accordance with 11.1.3,

13.1.5 The test organisms had not been cultured in the testing facility for at least the last two weeks before the test,

13.1.6 The test lasted less than ~~96~~ 72 h, unless a shorter duration has been shown to be acceptable with the test species, conditions, etc.

13.1.7 Cell counts in the controls did not increase by a factor of at least 16 during ~~t~~ a 72-h test period (corresponding to a specific growth rate of 0.9 day^{-1}),

13.1.8 Temperature and light fluence rate were not measured as specified in 11.8,

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

13.1.10 The light fluence rate did vary more than 15 %.

13.1.11 Exceptions to these requirements are, for example, if temperature was measured numerous times, a difference of more than 4°C might be inconsequential (see 13.1.8). However, if temperature was measured only a minimal number of times, one difference of more than 4°C might indicate that more such differences would have been found if temperature had been measured more often.

13.1.12 The coefficient of variation in the controls was greater than 20 % for yield or greater than 7 % for average specific growth rate.

13.2 *Other Indications of Test Acceptability* —It may be desirable to conduct periodic reference toxicant tests with microalgae.

Potassium chloride, zinc chloride, potassium dichromate, 3,5–dichlorophenol, or boron have been suggested. Each testing facility should maintain control charts for each algal species routinely tested to allow assessment of acceptable growth in controls and solvent controls under the test conditions.

14. Calculation

14.1 Depending on the method used to measure or estimate biomass (11.7), ~~three~~ several different IC50s can be calculated. For information on the merits of different ~~endpoints,~~ response variables, see **(23)** and **(24)** .

~~14.1.1 If biomass~~ It is ~~measured only at the end of the test in each test vessel, an IC50 can~~ recommended that two response variables be ~~calculated based on the~~ used: either final ~~standing crop.~~

~~14.1.2 If~~ biomass ~~is measured at least daily in each test vessel, IC50s can also be calculated based on growth rate~~ or ~~area under the curve, or both.~~ yield, and growth rate. Since IC50 values based upon different response variables will differ, it is important to identify the basis for each IC50. IC50 values based upon average specific growth rate will ~~usually~~ be ~~different from IC50s~~ higher than those based upon ~~area under~~ final biomass or yield due to the ~~curve, it~~ underlying mathematics. This is ~~important~~ not to ~~s~~ be interpreted as a difference in sensitivity between the response variables.

14.1.1 The initial biomass values are subtracted from the final biomass values in each test vessel to determine yield. It is acceptable (and actually preferable due to the difficulty in obtaining accurate estimates at test initiation) to use nominal biomass rather than measured biomass for the ~~calculation of~~ initial values. Since the ~~IC50.~~

~~14.1.2.1 If~~ initial values are extremely small compared to the final values, yield is essentially the same as standing crop (final biomass).

14.1.2 The average specific growth rate ~~(*u*)~~ is ~~used,~~ the ~~time interval used should be a period during which~~ growth rate calculated over the ~~appropriate control treatment(s) is in~~ entire test period. In addition, the ~~logarithmic phase~~ specific growth rate during the course of ~~growth. The interval~~ the test (days 0-1, 1-2, 2-3, etc.), also called the section-by-section growth rate, should be ~~either~~ calculated to assess effects of the ~~entire~~ test ~~period or a shorter period that does not include~~ material during the ~~initial (lag) phase or final (declining growth) phase~~ exposure period, such as an increased lag phase. Substantial differences between the section-by-section growth rates and the average specific growth rates indicate deviation from theoretical exponential growth and that close examination of the ~~control treatments, or both, if biomass in~~ data is warranted. In this instance, the ~~control treatment(s) increases by less than a factor~~ recommended approach is to compare specific growth rates from exposed cultures during time period of ~~two per day~~ maximum inhibition to controls during ~~these periods. The~~ the same time ~~interval should be used for each test vessel in all treatments. The~~ period.

14.1.2.1 The growth rate for each test vessel over the selected time interval is calculated as follows:

$$u = (\log_{10}(N_2/N_1))/t \quad (2)$$

$$u = (1n\, N_2 - 1n\, N_1) / t \quad (2)$$

where:

u = growth rate,
N_1 = biomass at the beginning of the selected time interval,
N_2 = biomass at the end of the selected time interval, and
t = elapsed time between the measurements of biomass, in days.

~~Alternatively,~~

Where the ~~growth rate can be calculated by converting~~ time interval is the ~~cell counts to their logarithmic values and regressing them against time;~~ entire test, the ~~slope of the regression line~~ resulting growth rate is the average specific growth rate.

~~14.1.2.2 If the area under the growth curve~~

14.2 It is ~~used, it should be calculated for~~ generally useful to tabulate the mean percent inhibition at each test ~~vessel as:~~ concentration, which is calculated as follows:

$$A = \frac{N_1 - N_o}{2} \times t_1 + \frac{N_1 + N_2 - 2N_o}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_a - 2N_o}{2} \times (t_n - t_{n-1}) \quad (3)$$

~~where:~~

~~A = area~~
~~N_o = nominal number of cells at time t_o,~~
~~N_1 = measured number of cells at time t_1,~~
~~N_a = measured number of cells at time t_a,~~
~~t_1 = time of first measurement after beginning of test, and~~
~~t_a = time of n^{th} measurement after beginning of test.~~

~~14.2 If percent reduction (*%I*) is used, it should be calculated for each test vessel in each treatment other than the control treatment(s), as follows:~~

$$\% I = \frac{C - X}{C} \times 100 \quad (4)$$

where:

C = the average value for the control test vessels, and

X = the value for an individual test treatment vessel or the average value for the treatment.

14.2.1 The IC50(s) and associated 95 % confidence limits are determined using appropriate statistical methodology (See Practice E 1847). Any or all of the following ~~(or percent inhibition values based on them)~~ may be used as the response variable: standing crop (final biomass); yield (final biomass minus initial biomass); average specific growth ~~rate; the area under the growth curves.~~ rate. The individual test vessel values should be used in fitting the regression model, not the mean for each concentration. The IC50 value(s) are calculated based upon the data generated for the entire ~~96-h~~ exposure period~~:~~ (typically 96 h); however, 24-, 48- and 72-h values may also be calculated, if desired. The model and the methodology for estimating the model parameters should be described. The results from the test of significance of the regression should be reported, along with one or more indicators of goodness of fit, such as the root mean square error or R-squared, or both. Estimates of the model parameters, for example, slope, may also be reported. One commonly used method for continuous data of this nature is weighted least squares nonlinear regression **(25)**. Other methods for modeling continuous data may also be used **(26,27)**.

14.3 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 causes 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 to 95 %.

14.4 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material caused a statistically significant inhibition in 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 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.

14.5 Other IC values may be calculated, such as IC10 or IC20.

14.6 The coefficient of variation for the controls for each response variable calculated should be presented.

15. Report

15.1 Include the following information on the record of the results of an acceptable ~~96-h~~ algal test, 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 the 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, name of person who identified the species and the taxonomic key used, and culture procedure used,

15.1.5 Description of the experimental design, test vessels and covers, volume of medium used in each test vessel, number of test vessels per treatment, and the lighting,

15.1.6 Average and range of the measured temperature and the methods of measuring or monitoring,

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

15.1.8 Method(s) used for measuring biomass, for example, cell counts, chlorophyll *a*, or dry cell weights,

15.1.9 A table of data on biomass of algae in each test vessel in the control(s) and the other treatment(s) in sufficient detail to allow independent statistical analysis,

15.1.10 Calculated IC50s, their 95 % confidence limits and calculation method(s) used; specify response variables used to calculate IC50s; 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 other relevant information.

15.2 Published reports should contain enough information to clearly identify the procedures used and the quality of the results.

16. Keywords

16.1 algae; algal-toxicity testing; aquatic plants; aquatic toxicity testing; microalgae

ANNEXES

(Mandatory Information)

A1. PREPARATION OF FRESHWATER MEDIUM (10)

A1.1 Add 1 mL of each stock solution in A1.2.1-A1.2.7 to approximately 900 mL of ~~deionized or distilled~~ ASTM Type I water and then dilute to 1 L.

A1.2 Macronutrient stock solutions are made by dissolving the following into 500 mL of ~~deionized or distilled~~ ASTM Type I water. Reagents A1.2.1, A1.2.2, A1.2.3, and A1.2.4 can be combined into one stock solution.

A1.2.1 $NaNO_3$—12.750 g.
A1.2.2 $MgCl_2 \cdot 6H_2O$—6.082 g.
A1.2.3 $CaCl_2 \cdot 2H_2O$—2.205 g.
A1.2.4 *Micronutrient Stock Solution* —(see A1.3).
A1.2.5 $MgSO_4 \cdot 7H_2O$—7.350 g.
A1.2.6 K_2HPO_4—0.522 g.
A1.2.7 $NaHCO_3$—7.500 g.
A1.2.8 $Na_2SiO_3 \cdot 9H_2O$—See Note A1.1.

NOTE A1.1—Use for diatom test species only. May be added directly (202.4 mg) or by way of stock solution to give 20 mg/L Si final concentration in medium. Some laboratories also add vitamins such as thiamine hydrochloride, biotin, and B12 to the medium for diatom test species.

A1.3 The micronutrient stock solution is made by dissolving the following into 500 mL of ~~deionized or distilled~~ ASTM Type I water:

A1.3.1 H_3BO_3—92.760 mg.
A1.3.2 $MnCl_2 \cdot 4H_2O$—207.690 mg.
A1.3.3 $ZnCl_2$—1.635 mg.
A1.3.4 $FeCl_3 \cdot 6H_2O$—79.880 mg.
A1.3.5 $CoCl_2 \cdot 6H_2O$—0.714 mg.
A1.3.6 $Na_2MoO_4 \cdot 2H_2O$—3.630 mg.
A1.3.7 $CuCl_2 \cdot 2H_2O$—0.006 mg.
A1.3.8 $Na_2EDTA \cdot 2H_2O$—150.000 mg.
[Disodium (Ethylenedinitrilo) tetraacetate].
A1.3.9 $Na_2SeO_4 \cdot 5H_2O$—0.005 mg **(268)** See Note A1.2.

NOTE A1.2—Use only in medium for stock cultures of diatom species.

A1.4 Adjust pH to 7.5± 0.1 with 0.1 *N* or 1.0 *N* NaOH or HCl.

A1.5 Filter the media into a sterile container through either a 0.22-µm membrane filter if a particle counter is to be used or a 0.45-µm filter if a particle counter is not to be used.

A1.6 Store medium in the dark at approximately 4°C until use.

A2. PREPARATION OF ENRICHED SALTWATER MEDIUM (~~27~~9)

A2.1 Prepare Stock Solution A by adding the specific amount of chemicals in the order listed below to 900 mL ~~distilled or deionized~~ ASTM Type I water and dilute to 1 L.

A2.1.1 *Metal Mix (A)*:
A2.1.1.1 $FeCl_3 \cdot H_2O$—0.048 g
A2.1.1.2 $MnCl_2 \cdot 4H_2O$—0.144 g
A2.1.1.3 $ZnSO_4 \cdot 7H_2O$—0.045 g
A2.1.1.4 $CuSO_4 \cdot 5H_2O$—0.157 mg
A2.1.1.5 $CoCl_2 \cdot 6H_2O$—0.404 mg
A2.1.1.6 H_3BO_3—1.140 g
A2.1.1.7 $Na_2EDTA.2H_2O$—1.0 g

A2.2 Prepare stock solution B by adding the specific amounts of the chemicals listed below to 900 mL ~~distilled or deionized~~ ASTM Type I water and dilute to 1 L.

A2.2.1 *Minor Salt Mix (B)*:
A2.2.1.1 $K_3 PO_4$—0.3 g
A2.2.1.2 $NaNO_3$—5.0 g
A2.2.1.3 $NaSiO_3 \cdot 9H_2O$—2.0 g

A2.3 Prepare Stock Solution C by adding the specified amount of chemicals in the order listed below to 900 mL ~~distilled or deionized~~ ASTM Type I water and dilute to 1 L.

A2.3.1 *Vitamin Stock Solution (C)* :
A2.3.1.1 *Thiamine Hydrochloride*—500 mg.
A2.3.1.2 *Biotin*—1 mg.
A2.3.1.3 B_{12} —1.0 mg.

A2.4 The stock solutions are added to a sterile container containing either natural salt water that has been filtered through a 0.22 µm membrane filter or reconstituted salt water. Add the amounts given below to prepare medium used for toxicity testing. Add twice the amounts given to prepare medium for use in maintenance of stock cultures.

A2.5 Add 15 mL of metal Stock Solution (A)/L of medium.

A2.6 Add 10 mL of minor salt Stock Solution (B)/L of medium.

A2.7 Add 0.5 mL of vitamin Stock Solution (C)/L of medium. Add 1 mL of vitamin mix if *Thalassiosira* is used.

A2.8 Adjust pH to 8.0± 0.1 with 0.1 *N* or 1.0 *N* NaOH or HCl.

A2.9 Store medium in the dark at approximately 4°C until use.

A3. PREPARATION OF COMPLETE SALTWATER MEDIUM (268) (PROVASOLI'S ASP 12)

A3.1 Prepare individual stock solutions by dissolving the following reagents into 1 L of ~~deionized or distilled~~ ASTM Type I water.

Reagents

A3.1.1 *NaCl*—280 g.
A3.1.2 *KCl*—7 g.
A3.1.3 $MgSO_4 \cdot 7H_2O$— 70 g.
A3.1.4 $MgCl_2 \cdot 6H_2O$— 40 g.
A3.1.5 *Nitrilotriacetic Acid (NTA)* —1 g (see Note A3.2).
A3.1.6 *Tris*—10 g.
A3.1.7 $CaCl_2 \cdot 2H_2O$— 14.62 g.
A3.1.8 Na_2 *glycerophosphate*$\cdot 5H_2O$ —1 g.
A3.1.9 $NaNO_3$—10 g.
A3.1.10 $K_3 PO_4$—1 g.
A3.1.11 $Na_2SiO_3 \cdot 9H_2O$ —5 g.

Metals (see Note A3.1)

A3.1.12 Na_2EDTA—1 g.
A3.1.13 $FeCl_3$—30 mg (see Note A3.3).
A3.1.14 $H_3 BO_3$—1.144 g.
A3.1.15 $MnCl_2 \cdot 4H_2O$— 144.08 mg.
A3.1.16 $ZnCl_2$—10.425 mg.
A3.1.17 $CoCl_2 \cdot 6H_2O$— 4.039 mg.
A3.1.18 *NaBr*—1.288 g.
A3.1.19 $SrCl_2 \cdot 6H_2O$— 608.8 mg.
A3.1.20 *RbCl*—28.26 mg.
A3.1.21 *LiCl*—22.14 mg.
A3.1.22 $NaMoO_4 \cdot 2H_2O$— 126.1 mg.
A3.1.23 *KI*—1.308 mg (see Note A3.1 and Note A3.2).
A3.1.24 $H_2 SeO_3$—1.634 mg (see Note A3.1 and Note A3.2).

Vitamins

A3.1.25 B_{12} —0.02 mg (see Note A3.2).
A3.1.26 *Biotin*—0.1 mg.
A3.1.27 *Thiamine*—1 mg.

A3.2 When assembling the medium add the following concentrations of stock solutions, in the order listed, into a sterile 1–L graduated cylinder: 100 mL, solutions A3.1.1-A3.1.7; 10 mL, solutions A3.1.8-A3.1.10; 30 mL, solution A3.1.11; 10 mL, solutions A3.1.12-A3.1.23; 2 mL, solution A3.1.24; and, 10 mL, solutions A3.1.25-A3.1.27. Dilute to 1 L with ~~distilled or deionized~~ ASTM Type I water.

A3.3 Adjust pH of the medium to 7.9 ± 0.1 with 0.1 *N* NaOH or HCl before autoclaving.

A3.4 Autoclave at 15 lb/in.^{2} for 25 min.

NOTE A3.1—These stock solutions can be made at 100× concentrations and diluted with ~~deionized or distilled~~ ASTM Type I water to achieve stock concentrations.

NOTE A3.2—Although not specifically included in the original medium, further work by Provasoli and his students suggested the following minimal changes:

~~(*1*) Increase in B_{12} concentration to 1 µg/L **(26)**;~~
(*1*) Increase in B_{12} concentration to 1 µg/L **(28)**,
~~(*2*) Addition of 2 µg/L selenium **(28, 29)**, and~~
(*2*) Addition of 2 µg/L selenium **(30, 31)**, and
(*3*) NTA addition, while not actually included in the original formulation of ASP_{12}, is suggested by Provasoli as a desirable growth additive at 100 mg/L.

NOTE A3.3—Dissolve in 25 mL concentrated HCl.

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