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Standard Guide for Conducting Renewal Phytotoxicity Tests With Freshwater Emergent Macrophytes[1]

This standard is issued under the fixed designation E 1841; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test guide is designed to give general guidance for assessing the potential phytotoxicity of water soluble test material to freshwater emergent macrophytes.

1.2 This renewal test continuously exposes selected plant species, growing in sediment, to various concentrations of test material, dissolved in a nutrient solution.

1.3 This test guide is based on the Toxic Substances Control Act (TSCA) guidelines for conducting toxicity tests with terrestrial

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plants (**1**)[2] and is applicable to most water soluble chemicals, either individually or in formulations, commercial products, or known mixtures (see Guides E 1193 and E 1598). With slight modifications the procedure also might be used for effluents (see Guide E 1192).

1.4 Results from this toxicity test can be used to report an E IC50 or NOEC (see Section 3) based on the concentration of chlorophyll extracted from the plants (see Guides D 3731 and E 1218). In some situations, it might be necessary to only test at one concentration to determine whether or not that specific concentration is toxic to the plants.

1.5 This test method is arranged as follows:

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1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:* [3]

D 3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters
E 729 Guide for Conducting Acute Toxicity Tests 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 Test on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians
E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*
E 1218 Guide for Conducting Static 96-h Toxicity Tests with Microalgae
E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing
E 1598 Practice for Conducting Early Seedling Growth Tests
E 1706 Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates
E 1733 Guide for Use of Lighting in Laboratory Testing

3. Terminology

3.1 The words "must," "should,"" may," "can," and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the

[2] The boldface numbers in parentheses refer to the list of references at the end of this test method.

[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 11.05. volume information, refer to the standard's Document Summary page on the ASTM website.

purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," is often "desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to,"" can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions*—For definitions of other terms used in this standard, refer to Terminology E 943 and Practice E 1598.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *EIC50*, *n*—a statistically or graphically estimated concentration of test material that, under specified conditions, is expected to cause one or more specified effects in 50 % of a group of organisms, for which the data are not dichotomous.

3.3.2 *emergent macrophyte*—vascular plant that typically has a well defined root system that anchors the plant in sediments and long linear erect leaves that emerge above the water surface.

3.3.3 ~~*NOEC*, *n*—the statistically or graphically estimated highest concentration of test material that, under specified conditions, is expected to cause no observable effects of a biological process, such as growth or reproduction, for which the data are not dichotomous.~~

~~3.3.4~~ *rhizome*, *n*—underground horizontal stems from which leaves and roots can develop.

3.3.54 *surrogate species*, *n*—plant species that may be used to gage or measure a response that might be demonstrated by another plant species exposed to similar conditions.

3.3.65 *tuber*, *n*—short, thickened, fleshy part of an underground stem, used for photosynthate storage.

4. Summary of Guide

4.1 Tubers, rhizomes or seeds of selected freshwater emergent macrophytes are planted in pots containing sediment.

4.2 The sediment is kept saturated constantly by placing the pots in trays that are kept filled with water so that the water level is below the rim of the pots. The plants are allowed to grow, and once firmly established, the phytotoxicity test may begin. Depending on the species and culture conditions this time period may be two to six weeks.

4.3 Pots containing the actively growing plants are placed in individual trays. This constitutes the test chamber. Each tray will contain a selected concentration of the test material dissolved in a nutrient solution. The amount of solution is not critical as long as there is a continuous supply. The test solutions including the control are renewed three times a week (see Guide E 1193).

4.4 Following a two-week exposure to the test solution, the plants are harvested by cutting the stems at the soil level.

4.5 To determine treatment differences, it is recommended that chlorophyll be extracted from the leaf material **(2)** and analyzed using High-Performance Liquid Chromatograph (HPLC). A spectrophotometer or fluorometer also may be used to determine treatment differences **(3-5)**.

4.6 A variety of procedures can be used to calculate the results of a growth test. Means comparison procedure can be used to determine if treatments are different from the control while regression may be used to determine ~~EC50s and NOEC.~~ IC50s.

5. Significance and Use

5.1 Increased emphasis is being placed on protecting wetlands **(6)** and several agencies including U.S. Environmental Protection Agency and Environment Canada are beginning to require, for the registration of pesticides, data regarding toxicity of test materials to rooted aquatic vascular plants **(7,8,9)**.

5.2 Much research is being conducted with vascular plants, both terrestrial and aquatic ~~**(9)**~~**(10)** , however, protocols for phytotoxicity testing with freshwater emergent macrophytes still are not well defined.

5.3 This guide is designed to assess potential detrimental effects of water soluble chemical substances on selected surrogate species of freshwater emergent macrophytes.

5.4 This guide focuses on diminishment of chlorophyll content in leaves as the measurable endpoint, however, not all chemicals affect chlorophyll production. Dry weight can be used as the endpoint for *O. sativa*, however, exposure times may need to be extended to detect treatment differences. Dry weight is not a recommended endpoint for any of the test species started as rhizomes or tubers. Other endpoints, such as peroxidase activity **(101)** or chlorophyll fluorescence **(12)** could possibly be used.

5.5 This guide could be used to provide early indication of potential problems, identify hazardous substances before contamination of wetlands occurs, and establish "margins of safety" for specific chemicals within wetlands (see Guide E 1023).

5.6 This guide is not designed to replace field assessments or other aquatic testing procedures. It is designed to compliment such testing, so that a more complete assessment is possible.

6. Apparatus and Reagents

6.1 *Facilities*—Plants are cultured and tests are conducted in areas where light and temperature can be controlled. A greenhouse or culture room is preferable. Light can be provided either by natural sunlight, fluorescent/incandescent lights or a mixture of both (see Guide E 1733). With the design of the test chambers having open water, humidity around the plants should be adequate for plant growth. To minimize interference, such as drafts, the plants can be shielded with curtains or partitions. Testing facilities should be kept separate from culturing facilities to prevent cross contamination.

6.2 *Test Chambers*—Plastic pots with drainage holes in the bottom are used for culturing and exposing the plants in the

phytotoxicity test. Pots should be large enough to prevent the plants from becoming root bound. Each pot is placed in an individual test tray that is larger in diameter than the pot and can hold the test solution.

6.3 *Cleaning*—The pots and test trays containing the plants should be disposable. All other equipment, except plastic, that will come in contact with the test solutions should be washed with a mild detergent and rinsed with water, a water-miscible organic solvent, water, acid, such as 10 % concentrated hydrochloric acid, and at least twice with ~~deionized, distilled, or dilution~~ ASTM Type I water.

6.4 HPLC—A system capable of performing binary or ternary linear gradients at a constant flow rate and capable of injecting 50 to 200 µL aliquots is recommended. The system should have a stainless steel HPLC column, packed with 5-µm C-18 reverse-phase packing and a column flow rate of 150 µL/min for a 250-mm long by 4.6-mm inside diameter column. For columns with different dimensions, the flow rate should be adjusted appropriately. The absorbance detector should be capable of detecting light in the visible region (400–700 nm). A data system or integrator for measuring peak areas is recommended as well.

6.5 *Reagents*:

6.5.1 *Dimethylsulfoxide (DMSO)*, solvent grade.

6.5.2 *Chlorophyll Standard*—Chlorophyll *A* from spinach prepared in DMSO (see 12.3.10).

6.5.3 *Water for HPLC Analysis*—HPLC grade or obtained from a water purification system capable of producing water with a resistivity > 12 mΩ/cm. Filter and degas (by vacuum or helium purging) before use.

6.5.4 *Ethyl Acetate*, HPLC grade. Filter and degas (by vacuum or helium purging) before use.

6.5.5 *Methanol*, HPLC grade. Filter and degas (by vacuum or helium purging) before use.

7. Hazards

7.1 It is recommended that the material safety data sheet (MSDS) be reviewed for safety, storage, and disposal precautions for each test substance.

7.2 Many materials can affect humans adversely if precautions are inadequate. Contact with all test materials and solutions, therefore, should be minimized by wearing protective gloves, especially when washing equipment or putting hands in test solutions, laboratory coats, aprons, glasses, and respirators if necessary. Information on toxicity to humans **~~(11-15)~~(13-17)**, recommended handling procedures **~~(16-19)~~(18-21)**, and chemicals and physical properties of the test material should be studied before a test is started.

7.3 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.

7.4 Cleaning of equipment with a volatile solvent, such as acetone, should be performed only in a well-ventilated area where no smoking, open flame, such as a pilot light, or sparking electrical equipment are present.

8. Nutrient Solution

8.1 The nutrient solution is one-half strength Hoagland's solution (Appendix X1) and is prepared by adding specified stock solutions to ASTM Type I water or other dilution water.

8.2 It is preferable to prepare the nutrient solution from ASTM Type I water. Alternatively, a constant source of dilution water, acceptable to the test organisms and available in adequate supply, should be used to make the Hoagland's solution. The minimal requirement for an acceptable dilution water is that healthy test species survive through germination, growth, and testing without showing signs of stress.

8.3 The quality of water from a well or spring usually is more uniform than surface water. Distilled or deionized water also is acceptable. Chlorinated water should not be used as the dilution water because it may be toxic to the plants. Dechlorinated, municipal drinking water should be used only as a last resort because the dechlorination process often is incomplete, and because the water may contain unacceptably high concentrations of copper, lead, zinc, and fluoride.

8.4 The water source should be analyzed several times a year (see Guide E 729) for physical and chemical factors including metals and other inorganic chemicals, and organic chemicals including pesticides. The concentrations in the dilution water should be below detection limit or the lowest concentration that has been shown to adversely affect the test species **(2~~0~~2)**.

9. Test Material

9.1 *General*—The test material should be reagent-grade or better, unless a test on a formulation, commercial product, or technical-grade material specifically is needed. Before a test is initiated, the following information should be obtained about the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, that is, impurities constituting more than 1 % of the material.

9.1.2 Solubility and stability in dilution water.

9.1.3 Potential for microbial degradation, transformation, sorption etc., of the test substance within the sediment matrix (see Test Method E 1706).

9.1.4 An estimate of toxicity to the test species. A range-finding study may be required.

9.1.5 Precision and bias of the analytical method at the planned concentration(s) of the test material.

9.1.6 Estimate of toxicity to humans and other organisms.

9.1.7 Recommended handling procedures (see Section 7).

9.2 *Test Concentrations*:

9.2.1 Chemical concentration are expressed by weight of test material per volume of nutrient solution. It is preferable to add the test material directly by weight to the nutrient solution; however, a stock solution, with or without a solvent, may be prepared (see 9.3) and appropriate aliquots added to each test solution.

9.2.2 To minimize variation, it is recommended the test solutions be made in batch, then equally distributed to individual test chambers.

9.2.3 The concentration of test material in each treatment should be measured at least at the beginning of the test and in the fresh renewal solutions. It is preferable also to measure the concentrations at the end of each renewal period. Test solutions may be pooled across replicates for each treatment.

9.2.4 Within each treatment, the highest measured concentration, in fresh test solutions, divided by the lowest concentration must be less than two. The variability of the sampling and analytical procedures should be determined before the beginning of the test to determine how may samples should be taken and analyses performed at each sampling point to ensure that this requirement is not violated just because of sampling or analytical variability.

9.2.5 The number of selected concentrations should be based on the goal of the study (see Section 12). Multiple concentrations can be used to calculate ~~E~~ IC50 or NOEC values. In some situations testing at a single concentration may be desirable (see Section 9.2.8).

9.2.6 If the test is intended to allow calculation of a ~~E~~ IC50 or NOEC value (see Section 12), the test concentrations should bracket the predicted ~~E~~ IC50 or NOEC value. The prediction might be based on the results of a test on the same or a similar test material with the same or similar test organism. If a prediction is not available, it usually is 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.

9.2.7 Concentrations exceeding water solubility should be considered for the test because aquatic macrophytes may sometimes be exposed to concentrations above water solubility and because solubility in dilution water often is not well known (see 9.3). The use of concentrations that are more than ten times greater than water solubility may not be worthwhile. With some test materials it might be found that concentrations above water solubility do not affect survival or growth any more than does the concentration that is at the water solubility limit.

9.2.8 When the object of the test is to determine the effect of a specific concentration of test material on the growth of the test species or whether or not the ~~E~~ IC50 or NOEC value is above or below a specific concentration, only that one concentration (see 12.1) and the controls (see 9.4) need to be tested.

9.2.9 The pH of the test solution should be measured in the highest, middle, and lowest test concentrations and in the controls at the beginning of the test and in both the fresh and used solutions at renewal. Other physical parameters, such as water ~~hardness, conductivity, dissolved oxygen,~~ hardness and ~~salinity~~ conductivity also may be ~~measured. Parameters should be adjusted only at the request of the researcher.~~ measured.

9.3 *Stock Solution*:

9.3.1 For test materials with low water solubility, a solvent can be used to make a stock solution that can be added to the nutrient solution.

9.3.2 If a solvent is necessary, its concentration in test solutions should be kept to a minimum and should be low enough that it does not adversely affect either survival or growth of the test organisms. When a solvent is used, a solvent control must be employed in the test (see 9.4). If an organic solvent is used, it should be reagent-grade or better, and its concentration in any test solution should not exceed 0.1 mL/L. 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.

9.3.3 If the concentration of solvent is not the same in all test solutions that contain test material or has unknown toxicity to the test organisms, a solvent test must be conducted to determine whether either survival or growth of the test species is related to the concentration of solvent over the range used in the phytotoxicity test, or a solvent test already must have been conducted using the same dilution water and test species. If either survival or growth is found to be related to the concentration of solvent, a test with that species in that water is unacceptable if any treatment contained a concentration of solvent in that range. If neither survival nor growth is found to be related to the concentration of solvent, a toxicity test with that same species in that same water may contain solvent concentrations within the tested range, but the nutrient-solvent control (see 9.4.3) must contain the highest concentration of solvent present in any of the other treatments.

9.4 *Controls*:

9.4.1 If no solvent other than water is used, then only a nutrient solution control must be included in the test.

9.4.2 If a solvent other than water is used, at least two controls must be included in the test. One would be the nutrient solution alone, and the second would be the nutrient solution to which the solvent, from the same batch used to make the stock solution, would be added.

9.4.3 The concentration of the solvent in the nutrient-solvent control should be equivalent to the highest concentration used in the test chemical solutions.

9.4.4 The percentage of organisms that show signs of stress, such as chlorosis, necrosis, etc., must be 10 % or less for each type of control.

9.4.5 At this time, no reference toxicants or positive controls are recommended.

10. Sediment

10.1 A standardized formulated sediment should be used ~~**(21, 22)**~~**(23, 24)**.

10.2 A natural sediment may be used; however, it first should be determined if plant growth and response to selected chemicals is similar in both the natural sediment and an formulated sediment.

10.3 The sediment should not have been exposed to any prior treatments and should be free of any contamination that may impact plant growth.

10.4 Information should be known about the sediment, such as particle size distribution, pH, percent total organic carbon, cation exchange capacity (see Guide E 1391).

11. Test Organisms

11.1 *Recommended Species*—It is recommended that a surrogate test species, *Oryza sativa* (domestic rice) be used. *Oryza sativa* is readily available and can be cultured easily to give uniform plants within a two-week time period.

11.2 *Alternative Species*—Other test species may be tested, but more research is needed to confirm their usefulness. Other species that are recommended for further study because they are readily available have been cultured successfully in the laboratory and are important wetland species ~~**(23, 24)**~~ **(25, 26, 27)** include:

11.2.1 *Dicotyledonae—Polygonum muhlenbergh*—(nodding smartweed) grows well in wet soils or shallow waters.

11.2.2 *Monocotyledonae—Phalaris arundinacea* (reed canary grass) grows best on moist lowlands. *Scirpus acutus* (hardstem bulrush) grows in either wet soils or shallow waters. *Spartina pectinata* (prairie cordgrass) grows in damp soil.

11.2.3 Although the above species may not be the most sensitive species, their use is encouraged to increase comparability of results.

11.2.4 Because the sensitivities of these species may differ substantially depending on the type of chemical and the nature of the exposure, it is desirable to conduct tests with two or more species from different families.

11.3 *Culturing*:

11.3.1 *Oryza sativa (O. sativa)* are obtained as seeds and can be kept in a cool area for one year. Seed germination can decrease with time and should be checked.

11.3.2 Alternate test species are often received as field collected root stock in the form of tubers or rhizomes and should be planted as soon as possible. They could be held for one to two weeks in a cool, moist environment. Some alternate test species can and should be obtained as seed.

11.3.3 Plants started from seed (that is *O. sativa*) must be the same age and from the same source. For field collected test organisms, care should be taken to collect plants that are approximately the same age and from the same area.

11.3.4 Plastic pots, containing equivalent amounts of sediment are used for growing and testing the plants. Pots should be large enough to prevent the plants from becoming root-bound. For *O. sativa*, the recommended pot size is a minimum of 5 cm in diameter.

11.3.5 Seed or root stock are planted in moist sediment, following the instructions from the supplier. With *O. sativa* , several seeds (up to 20) are sown, then later thinned to four plants/pot. When tubers and rhizomes are used, one to four plants, depending on their initial size, are placed into each pot.

11.3.6 Test pots are maintained in trays (see 6.2) that are kept partially filled with either dilution water or nutrient solution (see 8.1 and 8.2).

11.3.7 Plants should be maintained in a greenhouse or growth chamber with a minimum photoperiod of 16 h. Light intensity, measured at several locations at the plant canopy, should be maintained at a minimum of 30–40 W m^{-2} (about 150–200 µmol m $_{-2}s^{-1}$) and should not vary more than 20 %. Temperature should be maintained between 20° and 30°C.

11.3.8 After two weeks the *O. sativa* plants are ready for testing (plants should be approximately the same size, that is, 8 to 10 cm tall). For the alternate species, testing should begin once adequate new growth is noted. For monocotyledonous macrophytes, this may be a linear extension (greater than 10 cm) of one to three blades. For dicotyledonous macrophytes, this may be the development of five to seven leaves. Depending on the species this may take three to six weeks to achieve.

12. Procedure

12.1 *Experimental Design*—Decisions concerning aspects of experimental design, such as the dilution factor, number of treatments, and number of test chambers should depend primarily on the purpose of the test and the type of procedure that is to be used to calculate results. One of the following two types of experimental designs probably will be appropriate in most cases.

12.1.1 A growth test intended for the calculation of treatment differences (~~E~~IC50 or NOEC) based on a measurable endpoint usually consists of one or more controls and a geometric series of at least five concentrations of test material. Controls, in which the plants are not exposed to the test chemicals, must consist of a nutrient solution control and if necessary, a nutrient-solvent control (see 9.4). Except for the control(s) and highest concentration, each test concentration should be at least 50 % 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 0.5, five properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too high or too low. If the estimate of toxicity is particularly nebulous (see 9.2.5), six or seven concentrations might be desirable.

12.1.2 If it is necessary only to determine whether a specific concentration reduces survival or growth and the determination of an E IC50 or NOEC value is not required (see 9.2.8), then only that concentration and the control(s) are necessary. Two additional concentrations, at about one-half and two times the specific concentration of concern, however, are desirable for increased confidence in the results.

12.1.3 The minimum number of test chambers should be based on the expected variance between test chambers, and either the maximum acceptable confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing.

12.1.4 It is recommended that a minimum of five replicate chambers be used. Because of the importance of the controls in the calculation of results, it might be desirable to use more test chambers for the control treatment(s) than for each of the other treatments.

12.2 *Beginning of Test*

12.2.1 The testing location should be kept separate from the culturing location to prevent any cross contamination.

12.2.2 The pots containing the actively growing plants are transferred to new individual trays, one pot per tray. This then becomes the test chamber.

12.2.3 The trays are kept partially filled with nutrient solution to which the appropriate amount of test material (see Section 9) has been added. Enough nutrient solution should be added to the trays so that the sediment stays saturated, but the sediment surface is not covered. This helps control algal contamination on the sediment surface.

12.2.4 The test chambers are placed in a randomized complete block pattern (with each treatment being present in each block) and maintained under conditions similar to those used to culture the plants (see 11.3.6).

12.2.5 The test solutions, including the control, should be renewed three times a week. At this time, trays should be rinsed and any excess algal, bacterial or fungal contamination on the trays removed.

12.2.6 After two weeks the plants are harvested by cutting the stems at the soil surface.

12.2.7 All plants growing in an individual test chamber are combined and analyzed as one replicate.

12.3 *Evaluation of Test*:

12.3.1 Grind the plants by placing the tissue in a blender with dry ice. The amount of dry ice is not critical, however, it is recommended that approximately 250 mL of dry ice be used for 2 to 3 g (wet weight) of plant tissue. Plants will grind easier if they are first cut into smaller pieces.

12.3.2 To extract the chlorophyll, four subsamples (similar in wet weight) of the homogenous, ground plant tissue from each replicate are measured out. The weight for each subsample does not need to be exact because the calculations are based on dry weights.

12.3.3 Three of the subsamples are placed in Eppendorf tubes to which solvent is added. DMSO is the recommended solvent, however, dimethyl formamide (DMF) could also be used **(258)** . Acetone is not recommended due to incomplete extraction of the chlorophyll. The mixtures are vortexed for 30 s then centrifuged for 2 min before the supernatant is decanted into amber vials, which can be sealed.

12.3.4 Repeat the extraction procedure for each subsample two more times, combining the supernatant from the three extractions.

12.3.5 All extracts must be kept cool (0° to 4°C) and in the dark.

12.3.6 The stability of the extracted chlorophyll is limited, therefore, only extract the number of samples that can be analyzed in a 24 h time period.

12.3.7 To get dry weights, the fourth subsample from each replicate is first weighted then dried at 65°C for 48 h **(269)**. A wet:dry ratio is established and used to back-calculate the dry weights for the other subsamples.

12.3.8 Chlorophyll standards are prepared for each batch of extracts to be analyzed.

12.3.9 Prepare stock solutions of chlorophyll *A* by adding 2 mL of DMSO (or DMF) to 1 mg of commercially available chlorophyll *A*.

12.3.10 Five standards then are prepared by adding the appropriate amount of chlorophyll *A* stock to DMSO (or DMF).

12.3.11 The concentrations for the standards should bracket the suspected test concentrations.

12.3.12 It is recommended that a matrix spike (that is, sample from one of the highest test concentrations spiked with a known standard) and blank also be prepared.

12.3.13 When preparing stock solutions, standards and spikes, amber vials should be used and the preparation should be in a darkened room. Stocks and standards can be divided into small aliquots and maintained in the freezer for at least one week. Stocks and standards should be thawed only one time.

12.3.14 The extracts can be analyzed using a HPLC (see 6.4) at wavelength of either 433 nm or 668 nm with the following HPLC conditions: mobile phase *A* is 15/65/20 ethyl acetate/methanol/water (v/v/v); and mobile phase *B* is 60/30/10 (v/v/v). See Section 4.5 for other analytical techniques.

12.3.15 The solvent program for the HPLC is as follows: 100 % *A* for 0.2 min, linear gradient to 100 % *B* in 8 min, hold 12

min, return to 100 % *A* in 1.5 min. Equilibrate the column at 100 % *A* for a minimum of 10 min between samples and a minimum of 20 min prior to the first run after a shutdown period.

12.3.16 Plot "peak area" for the standards against the concentrations of the standards. Fit the data to a linear least squares model to obtain the slope and intercept.

12.3.17 Using this information, calculate the concentration of chlorophyll *A* (chl *A*) in the test extracts:

$$\mu g \text{ chl A/mL DMSO} = \frac{y - b}{m} \quad (1)$$

where:

y = peak area,
m = slope, and
b = intercept.

12.3.18 Correct for dry weight:

$$\mu g \text{ chl } A/\text{dry wt.} = \frac{\mu g \text{ chl } A}{\text{wet wt.}} \frac{\text{wet wt. (g)}}{\text{(g) dry wt. (g)}} \quad (2)$$

12.3.19 Mean concentrations of chlorophyll *A*/g of dried plant material then can be used to calculate treatment differences (see Section 13).

13. Calculation

13.1 Depending on the data to be analyzed, a variety of procedures can be used to calculate the results of a growth test.

13.2 The data also may be examined for the presence of outliners through the use of scatter plots or histograms. A probabilistic analysis also may be performed by running a randomized complete block analysis of variance and examining the studentized residuals **(27)(30)**. The presence of outliners may indicate a need for nonparametric analysis.

13.3 The treatments can be compared to the control using an appropriate means comparison procedure, such as a Dunnett's test, either one-tailed (if only low or high levels of the variable being analyzed are of interest) or two-tailed (if both high and low levels of the variable being analyzed are of interest). The error term used in the means-comparison procedure is derived from an appropriate analysis of variance, namely, randomized complete block with the test chamber (not individual plants within the test chamber) as the experimental unit. The overall significance of the *F*-test from the analysis of variance is not as important because the means comparison procedures (for example, Dunnett's test) control the overall level of significance for the number and type of comparisons actually performed. The 0.05 level of significance is suggested. The highest concentration not significantly different from the control is designated the non-observed-effect-concentration (NOEC).

13.4 Parametric analysis of variance is robust against departures from normality and differences in the amount of variability within each treatment level. To check these assumptions for a randomized complete block model. Departures from normality may be investigated by computing a statistic, such as the Shapiro-Wilk test. The homogeneity of variance across treatment groups may be tested using a statistical test, such as Levene's **(28)(31)**. If the *P* values for the test for normality or the test for homogeneity of variance, or both, is less than 0.01, conduct a nonparametric analysis. If neither of the *P* values is less than 0.01, conduct a parametric analysis. The results for both the parametric and nonparametric analysis may be reported. The power and MDD (minimum detectable difference) or any ANOVA should be calculated and reported. **(32)**

13.5 If concentrations corresponding to specified percentage inhibitions from the control mean are desired (such as an E IC50), they may be obtained through use of an appropriate regression model **(33,34)**. The dependent variable is defined as percent inhibition with 0 % corresponding to the control mean and 100 % corresponding to a value of 0 for the variable being analyzed, that is, percent inhibition = (100 × (control – test chamber value)/control). The control value may be either a mean over all blocks or the control value for the same block. The percent inhibition values for each test chamber receiving a (noncontrol) treatment should be used. The type of model and estimation method should be described along with goodness of fit statistics, such as the root mean square error, R^2, or 95 % confidence intervals about the estimates, or a combination thereof.

13.6 If the test contains more than one control, such as nutrient solution and nutrient-solvent control, they should be compared and pooled if found not to be significantly different. The same analysis of variance procedures should be used as in 13.3 and all treatment groups should be included, as well as the two control groups. The only means comparison of interest, however, is between the two control group's means. This maintains the same amount of power as is present in the subsequent comparisons of treatment group means to the appropriate control group mean. The decision to pool control groups should be made by considering both whether the amount of difference between the two control groups is biologically important and interpretable, as well as whether the difference is statistically significant. The results for comparisons to more than one control group may be reported.

13.7 The statistical procedures and computer programs used should be described in sufficient detail so that the calculations can be replicated easily. The statistical assumptions of, and the rationale for, the procedures used should be reported.

14. Acceptability of Test

14.1 The test is considered unacceptable if one or more of the following occur:

14.1.1 All test chambers are not identical in size, shape, and composition.

14.1.2 Plants are not the same age (similar age for field collected plants) and from the same source.

14.1.3 A required nutrient solution control and nutrient-solvent control was not included in the test or the solvent significantly affected the growth of the test species.

14.1.4 Temperature and light were not maintained as specified in 11.3.6.

14.1.5 Ten percent or more of the control organisms demonstrated some form of stress (chlorosis, necrosis, loss of turgidity, etc.).

14.1.6 Variation within the control test chambers (nutrient-solvent control test chambers included) for chlorophyll *A* was more than 30 % of the mean.

15. Report

15.1 The record of the results of an acceptable emergent macrophyte phytotoxicity test should include the following information either directly or by reference to the appropriate documentation:

15.1.1 Name of test and investigator(s), name and location of laboratory, and dates and time of initiation and termination of test, as well as, the dates and time of the culturing of the test organisms.

15.1.2 For the test materials, the source, lot number, CAS number, composition (identifies and concentrations of major ingredients and major impurities) if applicable, and known physio-chemical properties of the test material. The identi~~f~~ty and concentration(s) of any solvent used should be reported.

15.1.3 For the dilution water, its source, chemical characteristics, such as pH, hardness, etc., a description of any pretreatment, and a description of any ~~pretreatment~~ chemical analysis to confirm the absence of pesticides, PCBs, toxic metals, etc.

15.1.4 For the test organisms, their source, scientific name, age, size, life stage, holding and acclimation procedures including a description of the culturing conditions in terms of light and temperature.

15.1.5 For the sediment, its source, composition, pH, particle size, percent organic carbon. Any sediment pretreatment or chemical analysis results should be reported.

15.1.6 Description of the experimental design, test chambers (size, shape, composition), number of test chambers per treatment, number and types of controls, and duration of test.

15.1.7 Description of the test conditions including how light, temperature and humidity are controlled and measured and the range of measured test conditions.

15.1.8 Schedule and methods for preparing test solutions.

15.1.9 Methods and results (with standard deviations or confidence limits) of physio-chemical analyses of water quality and test concentrations(s), including validation studies and reagent blanks.

15.1.10 Definition(s) of the effect(s) used for calculating ~~E~~ IC50 and NOEC values and a summary of general observations on other effects.

15.1.11 Table of data on the number of test organisms exposed and results after exposure for each treatment replicate, including the control(s), in sufficient detail to allow independent statistical analyses.

15.1.12 The ~~E~~ IC50 value (along with 95 % confidence intervals) and NOEC value, and the methods used to calculate them.

15.1.13 Anything unusual about the test, any deviations from these procedures, and any other relevant information.

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

16. Precision and Bias

16.1 The precision and bias, for the procedure in this guide is for determining phytotoxicity using freshwater emergent macrophytes, are being determined.

17. Keywords

17.1 chlorophyll; emergent macrophyte; phytotoxicity test; surrogate species

APPENDIX

(Nonmandatory Information)

X1. HOAGLAND'S SOLUTION

X1.1 Stock solutions are made by dissolving the compounds, listed in Table X1.1, into distilled water (or an equivalent). Trace elements can be combined into stock solution No. 6.

X1.2 To make one-half strength Hoagland's solution for use in testing, add specified amount of each of the stock solutions, listed in Table X1.1, to approximately 900 mL of the dilution water. Bring the volume to 1 L. Adjust to pH 6.5 with 1*N* KOH or 1*N* HCl.

REFERENCES

(**1**) Federal Register, Vol 50, No. 188, 1985. 797.2800 *Early Seedling Growth Toxicity Test*. 797.2850 *Plant Uptake and Translocation Test*.

(**2**) Inskeep, W. P., and Bloom, P. R., "Extinction Coefficients of Chlorophyll A and B in N,N-dimethylformamide and 80 % Acetone," *Plant Physiology* , Vol 77, 1985, pp. 483–485.

(**3**) Ronen, R., and Galun, M., "Pigment Extraction for Lichens with Dimethyl Sulfoxide (DMSO) and Estimation of Chlorophyll Degradation," *Environmental and Experimental Botany*, Vol 24, 1984, pp. 239–245.

(**4**) Goedheer, J. C., "Fluorescence Bands and Chlorophyll a Forms," *Biochimica et Biophysica Acta*, Vol. 88, 1964, pp. 304–317.

(**5**) Holm-Hansen, O., Lorenzen, C. J., Holmes, L. W., and Strickland, J. D. H.," Fluorometric Determination of Chlorophyll," *Journal of Conseil International for the Exploration of the Mer.*, Vol 30, 1965, pp. 3–15.

(**6**) U.S. Environmental Protection Agency, "Water Quality Standards for Wetlands: National Guidance," Office of Water Regulations and Standards, Washington DC, WH-585, EPA 440/S-90-011, 1990.

(**7**) U.S. Environmental Protection Agency, "Pesticide Assessment Guidelines, Subdivision J. Hazard Evaluation Nontarget Plants," Office of Pesticide and Toxic Substances, Washington DC, EPA 540/9-82-020, 1982.

(**8**) Environment Canada, "Draft Guidelines for Registration of Chemical Pesticides in Canada: Nontarget Plant Testing and Evaluation," Canadian Wildlife Service Technical Report Series No. 145, 1992.

(**9**) Lewis, P., Matsumura, F., Odiott, O. and Roberts, S.M., A Set of scientific Issues Being Considered by the Environmental Protection Agency Regarding: Review of Non-Target Plant toxicity Tests Under the North American Free Trade Agreement (NAFTA), FIFRA Scientific Advisory Panel Meeting, June 27-29, 2001, Arlington, Virginia, Last modified Jan. 7, 2003, http://www.epa.gov/scipoly/sap/2001/june/finaljune.pdf

(**10**) Wang, W.," Use of Plants for Assessment of Environmental Contaminants," *Reviews of Environmental Contaminant Toxicology*, Vol 126, 1992, pp. 87–127.

(**1011**) Byl, T. D., and Klaine, S. J., Peroxidase Activity as an Indicator of Sublethal Stress in the Aquatic Plant *Hydrilla verticillata* (Royle). In: Plants for Toxicity Assessment: Second Volume, ASTM STP 1115. ed. Gorsuch, J. W., Lower, R. R., Wang, W., and Lewis, M. A. American Society of Testing and Materials, Philadelphia, 1991, pp. 101–106.

(**12**) Marwood, C.A., Solomon, K.R., and Greenberg, B.M., ″Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polycyclic aromatic hydrocarbons,″ Environmental Toxicology and Chemistry, Vol 20, 2001, pp. 890-898.

(**13**) International Technical Information Institute, "Toxic and Hazardous Chemical Safety Manual," Tokyo, Japan, 1977.

(**1214**) Sax, N. I., "Dangerous Properties of Industrial Materials, 5th ed.," Van Nostrand Reinhold Co., New York, 1979.

(**1315**) Patty, F. A., ed., "Industrial Hygiene and Toxicology," Vol II, second ed., Inscience, New York, NY, 1963.

(**1416**) Hamilton, A., and Hardy, H. L., "Industrial Toxicology," third ed., Publishing Sciences Group, Inc., Action, MA, 1974.

(**1517**) Gosslin, R. E., Hodge, G. C., Smith, R. P., and Gleason, M. N., "Clinical Toxicology of Commercial Products," fourth ed., Williams and Wilkins Co., Baltimore, MD, 1976.

(**1618**) Green, N. E., and Turk, A., "Safety in Working with Chemicals," Macmillan, New York, 1978.

TABLE X1.1 Preparation of 50 % Hoagland's Solution

Solution Number	Compound	Stock Solution	Stock Solution/Liter Water
1	KH_2PO_4	13.60 g/100 mL	0.5 mL
2	KNO_3	10.10 g/100 mL	2.5 mL
3	$Ca(NO_3)_2 \cdot H_2O$	23.60 g/100 mL	2.5 mL
4	$MgSO_4 \cdot 7H_2O$	24.70 g/100 mL	1.0 mL
5	$Na_2EDTA \cdot 2H_2O$	1.21 g/100 mL	0.5 mL
6	$FeCl_3$	0.60 g/100 mL	0.5 mL
7	Trace elements		0.5 mL
	H_3BO_3	1.43 g/500 mL	
	$MnCl_2 \cdot 4H_2O$	0.91 g/500 mL	
	$ZnSO_4 \cdot 7H_2O$	0.11 g/500 mL	
	$CuSO_4 \cdot 5H_2O$	0.04 g/500 mL	
	$Na_2MoO_4 \cdot 2H_2O$	0.01 g/500 mL	

(1719) National Research Council, "Prudent Practices for Handling Hazardous Chemicals in Laboratories," National Academy Press, Washington, DC, 1981.

(1820) Walters, D. B., ed.," Safe Handling of Chemical Carcinogens, Mutagens, Teratogens, and Highly Toxic Substances," Ann Arbor Science, Ann Arbor, MI, 1980.

(1921) Fawcett, H. H., and Wood, W. S., eds., "Safety and Accident Prevention in Chemical Operations," second ed., Wiley-Interscience, New York, NY, 1982.

(2022) U.S. Environmental Protection Agency, "Quality Criteria for Water," Office of Water and Hazardous Materials, Washington DC, 1976.

(2123) U.S. Environmental Protection Agency, "Germination, Survival, and Production of Marsh Plant Seedlings in Pulp and Paper Mill Effluent and in Sediments from St. Andrews Bay, FL," Office of Research and Development, Gulf Breeze, FL, EPA/600/X-89/032, 1989.

(2224) Environment Canada, "Guidance on Measurement of Test Precision Using Control Sediments Spiked with a Reference Toxicant," third draft, Office of Conservation and Protection, Ottawa, Canada, 1994.

(2325) Walsh, G. E., Weber, D. E., Simon, T. T., and Brashers, L. K., Toxicity Tests on Effluents with Marsh Plants in Water and Sediment. *Environmental Toxicology and Chemistry* , Vol 10, 1991, pp. 517–525.

(2426) Notenboom, E., "A Photometric Assessment of Soil Fertility of River Marginal Wetlands in France, England and Ireland," University of Utrecht, Department of Plant Ecology and Evolutionary Biology Report No. 931102, 1993.

(2527) Fairchild, J.F., Ruessler, S.D., and Carlson, A.R., ″Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metolachlor,″ Environmental Toxicology and Chemistry, Vol 17, 1998, pp. 1830-1834.

(28) Moran, R., and Porath, D., Chlorophyll Determination in Intact Tissues Using N,N-dimethylformamide. *Plant Physiology*, Vol 65, 1980, pp. 478–479.

(2629) Walsh, L. M., and Beaton, J. D., eds., "Soil Testing and Plant Analysis," Soil Science Society of America, Inc., Madison, WI, 1973.

(2730) Belsey, D. A., Kun, E., and Welsch, R. E. , "Regression Diagnostics: Identifying Influential Data and Sources of Collinearity," John Wiley and Sons, NY, 1980.

(2831) Milliken, G. A., and Johnson, D. E., "Analysis of Messy Data, Vol 1, Designed Experiments" Van Nostrand Reinhold Company, New York, NY, 1984.

(32) Hanson, M.L., Sanderson, H.,and Solomon, K.R., ″Variation, replication and power analysis of *Myriophyllum* spp. microcosm toxicity data,″ Environmental Toxicology and Chemistry (in press 2003).

(33) Stephenson, G.L., Koper, N., Atkinson, G.F., Solomon, K.R., Scroggins, R.P., ″Use of nonlinear regression techniques for describing concentration-response relationships of plant species exposed to contaminated site soils,: Environmental Toxicology and Chemistry, Vol 19, 2000, pp. 2968-2981.

(34) Streiberg, J.C., Rudemo, M., and Jensen, J.E., ″Dose-response curves and statistical models,″ Herbicide Bioassays, ed. J.C. Streibig, and P. Kudsk, CRC Press, Boca Raton, FL, 1993, pp. 29-55.

(35) Hoagland, D. R., and Arnon, D. I., "The Water Culture Method for Growing Plants Without Soil," California Experiment Station, Circular 347, Berkeley, CA, 1950.

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