



Standard Guide for Conducting Renewal Phytotoxicity Tests With Freshwater Emergent Macrophytes¹

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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 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 EC50 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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² The boldface numbers in parentheses refer to the list of references at the end of this test method.

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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:

- D 3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters³
- E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians³
- E 943 Terminology Relating to Biological Effects and Environmental Fate³
- E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses³
- E 1192 Guide for Conducting Acute Toxicity Test on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians³
- E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*³
- E 1218 Guide for Conducting Static 96-h Toxicity Tests with Microalgae³
- E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing³
- E 1598 Practice for Conducting Early Seedling Growth Tests³
- E 1706 Test Methods for Measuring the Toxicity of

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



Sediment-Associated Contaminants with Fresh Water Invertebrates³

E 1733 Guide for Use of Lighting in Laboratory Testing³

3. Terminology

3.1 The words “must,” “should,” “may,” “can,” and “might” have very specific meanings in this guide. “Must” is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. “Must” is only used in connection with factors that directly relate to the acceptability of the test (see Section 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 *EC50, 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.

3.3.5 *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.

3.3.6 *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.

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 aquatic vascular plants (7, 8).

5.2 Much research is being conducted with vascular plants, both terrestrial and aquatic (9), 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 (10) 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 complement 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 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 $\mu\text{L}/\text{min}$ 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 $> 12 \text{ m}\Omega/\text{cm}$. 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), recommended handling procedures (16-19), 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 dilution water.

8.2 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 (20).

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