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Standard Guide for Conducting Static, Axenic, 14-Day Phytotoxicity Tests in Test Tubes with the Submersed Aquatic Macrophyte, *Myriophyllum sibiricum* Komarov [1]

This standard is issued under the fixed designation E 1913; 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 Submersed rooted aquatic macrophytes are important components of aquatic systems. They contribute to primary productivity, improve water quality, cycle nutrients, generate oxygen, affect flow patterns, provide habitat and food for other organisms, and stabilize the sediment. These plants can be adversely affected when pesticides are sprayed to control aquatic weeds and algal blooms or when phytotoxic chemicals enter the waterway through atmospheric fallout, soil erosion, industrial effluent, sewage discharge, spills or drift from aerial or ground applications.

1.2 This guide is designed to give guidance for assessing the potential phytotoxicity of a test material added to a sterile liquid growth medium on a certain species of freshwater submersed macrophyte (*Myriophyllum sibiricum* Komarov) during a 14-day static exposure. A sterile system is recommended to determine the direct effect of the test chemical upon individual parameters of the submersed macrophyte. Overall environmental impact can not be directly determined. These procedures could possibly be useful for conducting toxicity tests with other species of submersed macrophytes, although modifications might be necessary **(1-5)**[2].

1.3 The procedures in this guide are applicable to most chemicals, either individually or in formulations, commercial products, or known mixtures. These procedures might be used to conduct tests for dependency on temperature, light, nutrients and pH. With appropriate modification, these procedures might be used to conduct tests for contaminated surface waters and aqueous effluents (see Guide E 1192). This static, axenic toxicity test might not be applicable to materials that contain microorganisms unless the sample can be filter sterilized without removing the toxicant. If the test materials are highly volatile, care should be taken to ensure that the test chambers are isolated. It might be necessary to replace the test material on a regular basis if the test material is rapidly biologically or chemically transformed in aqueous solution, or is removed from the test solutions in substantial quantities by the test chambers or organisms during the test. This toxicity test is not suitable for testing interactions between aquatic plants and other organisms, such as plant pathogens.

1.4 Results from the toxicity test outlined in this guide can be reported in terms of a 14-day IC25, IC50, or NOEC. This parameter may be based on several endpoints including inhibition of plant growth during the 14-day period, inhibition of shoot length, inhibition of root number and length, inhibition of fresh or dry weight (see Guide E 1415), inhibition of oxygen production, change in membrane permeability, and change in chlorophyll *a,* chlorophyll *b* and carotenoid content extracted from sections of the plants (see Practice D 3731 and Guide E 1218), **(6-12)**. All or some of these endpoint parameters may be examined depending upon the mode of phytotoxic action or researcher preference. It might be necessary to conduct the toxicity test at only one concentration to determine whether or not that specific concentration is inhibitory to plant growth and development.

1.5 This guide is arranged as follows:

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

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1.6 The values stated in SI units are to be regarded as the standard.

1.7 *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.* This standard may involve hazardous materials, operations, and equipment. See Section 9 for specific hazard statements.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Water[3]

D 3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters[4]

D 3978 Practice for Algal Growth Potential Testing with *Selenastrum capricornutum*[4]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[4]

E 943 Terminology Relating to Biological Effects and Environmental Fate[4]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[4]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[4]

E 1218 Guide for Conducting Static 96-h Toxicity Tests with Microalgae[4]

E 1415 Guide for Conducting Static Toxicity Tests With *Lemna gibba* G3[4]

E 1598 Practice for Conducting Early Seedling Growth Tests[4]

E 1733 Guide for the Use of Lighting in Laboratory Testing[4]

E 1841 Guide for Conducting Renewal Phytotoxicity Tests With Freshwater Emergent Macrophytes[4]

E 1847 Practice for Statistical Analysis of Toxicity Tests Conducted under ASTM Guidelines[4]

3. Terminology

3.1 *Definitions:*

3.1.1 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 conditions, unless the purpose of the test requires a different design. *Must* is only used in connection with factors that directly relate to acceptability of the test (see Section 15). *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.*

3.1.2 For definitions of other terms used in this guide, refer to Terminology D 1129, Guide E 729, Terminology E 943, and Practices E 1598 and E 1847.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *apex*—the uppermost portion of a plant containing the actively growing tissue or apical meristem.

3.2.2 *axenic or sterile*—free from other organisms, both active and dormant.

3.2.3 *culture*—the stock of organisms that is raised under controlled conditions to produce test organisms through asexual reproduction.

3.2.4 *submersed macrophyte*—a rooted freshwater vascular plant that remains covered with water during the growing season.

3.2.5 *toxicity test*—a standardized procedure that measures the concentration at which a test material has a defined effect upon the test organism.

3.2.6 *turion*—an asexual reproductive structure formed on lateral branches in response to lower autumn temperatures. Turions develop into new plants when environmental conditions become favorable **(13-17).**

4. Summary of Guide

4.1 Axenically cultured aspices of *Myriophyllum sibiricum* are exposed in a static system to a single concentration or a dilution series of a test substance. At the end of a 14-day test period under standardized conditions, growth and development of plants exposed to the test material is compared with the growth and development of plants in an appropriate control. A test substance is considered biologically active when a statistically significant dose-dependent inhibition of plant growth occurs.

4.2 The axenic toxicity testing technique involves exposing the test organism to selected concentrations of the test chemical in individual tubes. Each test tube contains a rooting substrate and 40 mL of nutrient medium previously spiked with the test

[3] *Annual Book of ASTM Standards,* Vol 11.01.

[4] *Annual Book of ASTM Standards,* Vol 11.05.

chemical. In this axenic testing system, 3 g of Turface®[5] has proven successful as an artificial rooting medium **(6)**. Without a rooting substrate, the plants roots may push the plant segment upwards and out of the nutrient medium. The use of another rooting medium would need to be validated. A 3–cm apical segment of *M. sibiricum* is added to the tube. The tubes are incubated (16 h light (fluence rate = 100 – 150 µmol $m^{-2}s^{-1}$) and 8 h dark at 25°C during the light and 20°C during the dark phase) for 14 days, during which time the increase in plant height over time may be measured and growth curves established. On Day 14, other possible test endpoints that may be measured include final shoot length, root number and total root length, total fresh weight, chlorophyll *a*, chlorophyll *b* and carotenoid content, membrane integrity, and oxygen evolution.

5. Significance and Use

5.1 Protection of aquatic areas is currently being emphasized by several agencies including the U.S. Environmental Protection Agency and Environment Canada. For pesticide registration, these agencies are beginning to require data regarding the toxicity of test chemicals to aquatic vascular plants **(19-21)**.

5.2 Recently, toxicological research with terrestrial and aquatic vascular plants has been initiated **(22)** including the development of a protocol for testing with emergent macrophytes (Guide E 1841) **(23)**. However, protocols for phytotoxicity testing with freshwater submersed plants still require development. Toxicological research has been conducted using submersed macrophytes **(1-5, 24-28)** but standardization of the methods is required.

5.3 This guide is designed to assess the phytotoxic effects of chemicals upon a selected freshwater species of submersed aquatic macrophyte, *Myriophyllum sibiricum* Komarov. This species is an ecologically important submersed aquatic dicotyledon with a north temperate distribution. It is readily cultured in test tubes in the laboratory **(29)**. Lower temperatures in autumn initiate the formation of turions on lateral branches that develop into new plants when environmental conditions become favorable **(13-17)**. Toxicological testing with this species has demonstrated that it is an ideal species for laboratory testing since it grows readily under laboratory conditions, the toxic response is reproducible and there is very little variation between experimental replicates **(6-12)**.

5.4 It is a common practice to use sterile plant culture when testing the direct effects of test materials upon a plant species. Sterile plant culture and toxicity testing have been conducted with algae (Practice D 3978, **30-32**), floating aquatic macrophytes (Guide E 1415, **30, 33**) and submersed aquatic plant species **(2-5)**. An axenic testing system is designed to determine the direct effect of the test material upon the test species. There is nothing except the plant within the test system that could degrade or otherwise change the test chemical. Hydrolysis or phytolysis may occur but degradation studies can determine the rate of degradation by these means. Axenic tests are especially valuable during the initial stages of examining a new compound (for example, pesticide evaluation and registration (Tier 1 and Tier 2)) **(19-21)**. In studies with other species of aquatic macrophytes, it has been shown that the presence of filamentous algae can cause a reduction in new shoot growth, fresh weight and chlorophyll *a* content of the macrophytes when compared to macrophytes grown in the absence of algae **(34)**. The test tubes are recommended for testing because they require a small incubation area, small amount of plant tissue, small volume of test material and allow for the maintenance of a sterile culture **(2, 3, 29)**. Furthermore, test tubes permit height measurements *in situ* **(29)**.

5.5 There are numerous possible physiological and morphological endpoints that can be utilized to assess the toxicity of chemicals to this aquatic plant species. The test material effect is assessed as a change in total plant height, growth rate, fresh or dry weight, number and total length of roots, chlorophyll *a*, chlorophyll *b*, carotenoids, membrane integrity or oxygen evolution, or any combination of these parameters. Peroxidase activity might be another endpoint that could be explored **(24-28)**.

5.6 This toxicity test may be utilized during the pesticide registration process, to provide an early warning of potential ecosystem problems, identify hazardous chemicals before contamination of aquatic systems occurs, and help establish "margins of safety" for specific chemicals within wetlands (see Guide E 1023).

5.7 This test is not designed to replace field assessments of test material damage or other aquatic testing procedures, but should be used as a screening tool. It should compliment other testing so that a more complete environmental assessment is possible. It is difficult to interpret effects observed in the lab in reference to those that could be found in the environment. Currently, there is a need for additional field data to validate the results obtained in laboratory plant toxicity tests. Since this toxicity test can detect non-lethal physiological endpoints as well as morphological changes, this toxicity test could act as an early warning system for possible environmental effects. If effects are noted in this toxicity test, it could indicate that further lab and field testing may be required.

6. Interferences

6.1 Since this test is designed as an axenic testing system, there is the possibility of microorganism contamination that could render the test results invalid. This microorganism contamination can reduce the nutrient content of the liquid nutrient solution, interfere with light intensity and interact with the test chemical. During a test, contamination (bacterial, fungal, or algal) can be assessed by visually examining every test plant within each test system for the presence of a cloudy or fuzzy appearance that could indicate the presence of contamination. This contamination is usually evident within 6

[5] The sole source of supply of Turface® known to the committee at this time is Aimcor, Applied Industrial Materials Corporation, One Parkway North, Suite 400, Deerfield, IL 60015. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee [1] which you may attend. Turface® has been found satisfactory for the purpose of an artificial rooting medium. Other non-opaque rooting medium, such as silica sand or glass beads, allow too much light to reach the rooting area and the roots begin to store/produce photosynthetic pigments. Fine grained substrates, such as mineral soil, organic soil, natural sediment, or formulated sediment **(18)**, may reduce light penetration by adhering to the test tube or plant or by remaining suspended in the nuntrient medium **(6)**.

days but definitely by the end of the 14-day test period. To help identify any potential contamination, random sampling of plants and media should be conducted during stock plant transfer and test initiation. Place approximately 1 mL of liquid nutrient solution or an unused plant segment onto an agar plate (for example, trypticase soy agar (TSA), potato dextrose agar (PDA) or other suitable agar medium). Incubate for a minimum of seven days and microscopically or macroscopically check for bacterial or fungal contamination. Contaminated plants and media should be autoclaved and disposed. They must not be used in a test. Maintaining sterile test conditions ensures that any change in the condition of the test plant is the direct effect of the unaltered test material and is not caused by an interaction between the test material and the contaminating organism.

6.2 Autoclaving may cause precipitation of certain constituents in the liquid growth medium or test material and may change the pH **(33)**. The pH should be monitored after autoclaving and adjusted if necessary. These precipitates are not necessarily irreversible or unavailable as nutrients. Irreversible precipitation renders the growth medium or test material unusable, unless the precipitate and its effect on the test endpoint(s) are known. The test material, medium or constituents that precipitate upon autoclaving may be filter sterilized.

7. Apparatus

7.1 *Facilities*—Stock plants should be cultured in and experiments should be conducted in environmentally controlled growth chambers in which light and temperature can be manipulated. Culturing facilities should be isolated from the toxicity testing facilities to minimize the risk of stock culture contamination by volatile chemicals released from test solutions. Light should be provided by either fluorescent or incandescent lights or a mixture of both. If an application of the spectrum of natural sunlight is required, see Guide E 1733. Minor changes in humidity are not of concern because the plants are within a closed system.

7.2 *Test Chambers*—All sterile stock and test plants are maintained in borosilicate glass test tubes (15 by 2.5 cm in diameter) (see Fig. 1). For stock plants, each test tube is covered with a clear plastic test tube closure (inside diameter (I.D.) = 25 mm, 38 mm in height). The test tubes are recommended for testing because of the small area required for incubation and the ease in maintaining a sterile culture. The test tube culture also encourages a vertical growth habit that facilitates height measurements during the test run. When the plant is being utilized for experimentation, each test tube contains a rooting substrate. Turface®[5] (3g) has proven successful in this axenic testing system. When culturing *Hydrilla verticillata*, a fine sand covered with a polytetrafluoroethylene barrier between two layers of agar has been used as an artificial rooting substrate **(3)**. Fine silica sand, glass beads, mineral soil, organic soil, natural field collected sediment and a formulated sediment **(18)** are not as effective for culturing *M. sibiricum* in this sterile test system **(6)**. After addition of the plant segment, the test chamber is covered with a clear plastic test tube closure (I.D. = 25 mm, 38 mm in height) fitted with a 35–mm section of tubing (I.D. = 7 mm, outside diameter (O.D.) = 10 mm) (nontoxic, nonabsorbent, autoclavable material such as clear polyethylene or other polymer tubing) glued with epoxy into the inside center of the closure. The tubing section supports a measuring rod (15–cm section of a Westergren blood sedimentation tube within the test chamber so that plant height measurements can be made during the 14-day test period. It is important to ensure that neither the tubing nor the epoxy glue contact the liquid nutrient solution, the test solution or the plant segment during the testing period.

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

7.3.1 *Autoclave*—To ensure that all dry material and liquid solutions are sterile, they must be autoclaved in an autoclave for 20 min, at 121°C and $1.31 \cdot 10^5$ Pa. The liquid cycle should contain a slow exhaust portion. Guide E 1218 recommends microwaving as an acceptable alternative to autoclaving and this technique has been used for the sterilization of soil **(35)** and phytoplankton culture medium **(36)**. Microwaving times and temperature cycles should be investigated before being used in this test method because microwaving might not acceptably eliminate the microbial population **(37)**.

7.3.2 *Laminar Airflow Cabinet*—All manipulations of plant material, liquid media and test solutions must be conducted within a sterile environment. A laminar airflow cabinet is most commonly used to maintain sterile conditions. The sterility of the cabinet must be maintained and the filters cleaned on a regular basis. A UV sterilization hood may also be acceptable but the sterility of the work space should be determined prior to experimentation.

7.3.3 *Analytical Balance*, capable of accurately weighing to 0.01 mg.

7.3.4 *Erlenmeyer Flasks*, borosilicate glass, numerous sizes.

7.3.5 *Fernbach Flasks*, borosilicate glass, 2800 mL.

7.3.6 *Volumetric Flasks*, borosilicate glass, various sizes.

7.3.7 *Micropipettes.*

7.3.8 *Volumetric Pipettes*, 1 to 50 mL, graduated, pipette bulbs and filters.

7.3.9 *pH Meter*, and calibrating solutions.

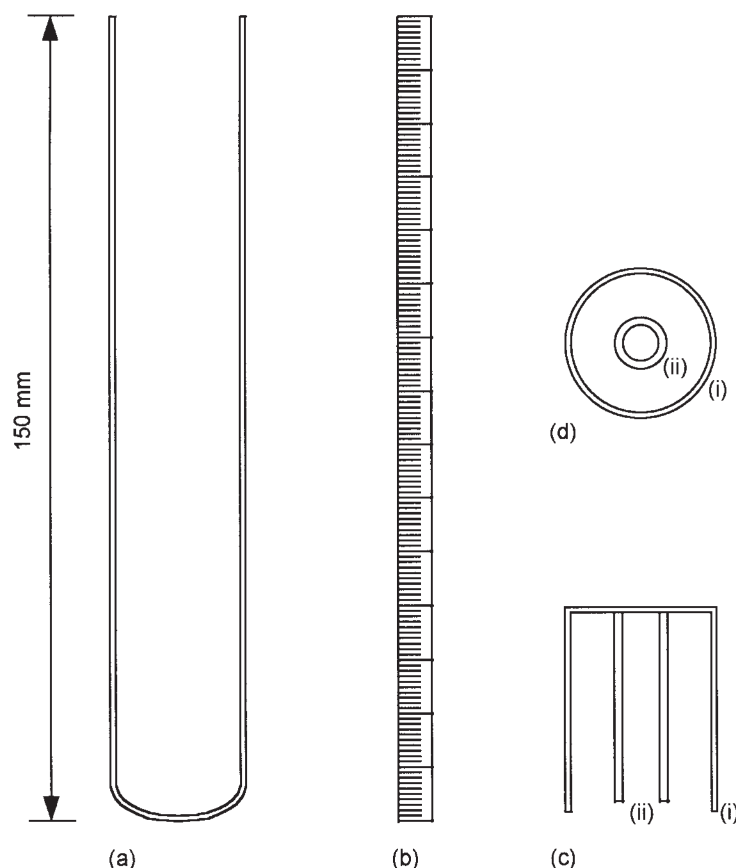
7.3.10 *Conductivity Meter*, and calibrating solutions.

7.3.11 *Light Meter*.

7.3.12 *Spectrophotometer*.

7.4 *Cleaning*—Test chambers and equipment used to prepare and store growth medium, stock solutions and test solutions must be cleaned thoroughly before and after use. Residues on the glassware can adversely affect the *Myriophyllum* growth. To remove all trace metals and organics, all reusable glassware (test tubes, Erlenmeyer, Fernbach and volumetric flasks, pipettes, etc.) should be cleaned in warm water with a non-phosphate detergent, triple rinsed with tap water, triple rinsed with deionized water, rinsed with 10 % HCl (v/v), rinsed three times with deionized water, rinsed with acetone, and triple rinsed with deionized water. Equipment should be dried, capped with appropriate closures or covered in aluminum foil and autoclaved for 20 min at 121°C and $1.31 \cdot 10^5$ Pa.

7.5 *Acceptability*—To determine the acceptability of new testing facilities, it is desirable to conduct a preliminary growth test, in which plants are grown in test chambers containing growth medium with no added test material. This is to



NOTE 1—At the start of an experiment, a 3 cm apical segment of plant plus a measuring rod are placed inside the test chamber. The tip of the measuring rod fits inside the polymer tube as the clear plastic tube closure is placed on top of the test chamber.

FIG. 1 (a) Test Chamber
(b) Measuring Rod - a 15 cm Section of a Westergren Blood Sedimentation Tube
(c) Clear Plastic Test Tube Closure (i) with Segment of Polymer Tube (ii) (Longitudinal View)
(d) Clear Plastic Test Tube Closure (i) With Segment of Polymer tube (ii) (Cross-Sectional View)

determine before the first toxicity test whether the plants will grow acceptably in the new facilities, whether the growth medium, handling procedures, sterility, etc., are acceptable, whether there are any location effects on growth, and the magnitudes of the within-chamber and between-chamber variances.

8. Reagents

8.1 Reagent grade (or better) chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[6] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. The test material should be reagent grade or better unless a test on a formulation, commercial product, or technical-grade material is specifically required.

8.2 References to water shall be understood to mean reagent water as defined by Type 1A or equivalent, as recommended in Specification D 1193.

9. Hazards

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

9.2 Many materials can affect humans adversely if precautions are inadequate. Therefore, contact with all test materials and solutions 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 **(38-42)**, recommended handling procedures **(43-47)**, and chemical/physical properties of the test material should be studied before a test is started.

9.3 Dispose stock solutions, test solutions, test organisms and artificial substrate in a manner appropriate to the test material. Health and safety precautions and applicable regulations should be considered before beginning a test. Removal or

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

degradation of test material might be desirable before disposal of stock and test solutions.

9.4 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.

10. Nutrient Solution

10.1 The nutrient solution is full strength modified Andrews' medium. It is prepared by adding specified amounts of nutrient salts to reagent water. Then, appropriate volumes of these nutrient salt stock solutions are added to reagent water (See Appendix X2) **(5)** to produce the liquid nutrient medium (modified Andrews' medium). This liquid nutrient solution is used for culturing stock plants, for growing the test plants and for preparing the test chemical solutions **(29)**. The nutrient medium must support healthy *M. sibiricum* growth through 14 days without the stock or control test plants showing signs of stress.

10.2 A constant source of reagent water, acceptable to the test organism and available in adequate supply, should be used to make the modified Andrew's medium. The reagent water and nutrient solution must be free of microorganisms after autoclaving.

10.3 Chlorinated or dechlorinated municipal tap water should not be used as the reagent water because it may be toxic to the plants.

10.4 The water source should be analyzed semi-annually (see Guide E 729) for physical and chemical factors including metals and other inorganic chemicals, and organic chemicals including pesticides. The concentration of these potentially harmful factors in the reagent water should be below the detection limit or the lowest concentration that is adversely toxic to the test species **(47)**.

11. Test Material

11.1 *General*—The test material should be reagent grade or better, unless a specific formulation, commercial product, or technical grade material is under examination. Before a test is initiated, the following information should be obtained about every test material:

11.1.1 Identities and concentrations of major ingredients and major impurities (that is, ingredients or impurities constituting more than 1 % of the material).

11.1.2 Solubility and stability of the test material in the reagent water and nutrient solution.

11.1.3 Stability of the test material if autoclaving or filter sterilization is required.

11.1.4 An estimate of the test material toxicity to the test organism under the conditions of this guide. A preliminary range-finding test may be conducted.

11.1.5 Precision and bias of the analytical method at the concentration(s) of test material to be tested.

11.1.6 Estimate of human toxicity and the toxicity to other organisms.

11.1.7 Recommended test material handling procedures (see Section 9).

11.2 *Test Concentrations*:

11.2.1 Chemical concentrations are expressed by weight of the test material per volume of nutrient solution. For each test concentration, the correct amount of test chemical may be added directly to the nutrient solution. A stock test chemical solution made with nutrient solution or a solvent may also be prepared and appropriate aliquots added to the different test dilutions.

11.2.2 The concentration of test material in each treatment should be measured at least at the beginning and end of the test. If the test is modified so that the test solution is renewed during the 14-day test period, the concentration of test material in the original medium and replacement solution may also be determined. Test solutions may be combined from the different replicates within each treatment.

11.2.3 The number of selected test concentrations should be based upon the study goals (see Section 13). Testing at a single level or multiple concentrations may be conducted. Multiple concentrations allows for the calculation of an IC25, IC50, or NOEC value but a percent inhibition may still be calculated if only one test concentration is used.

11.2.4 If calculation of an IC25, IC50 or NOEC value (see Section 13) is anticipated, the test concentrations should bracket the expected IC25, IC50, or NOEC value. The expected value might be based upon the results of a test on the same or a similar test material with the same or a similar test organism. If there are no literature values available, then it is desirable to conduct a range-finding test in which the test species is exposed to the control and three to five concentrations of the test material that differ by a factor of ten. As the similarity between the range-finding test and the actual test increases, the more useful will be the information obtained from the range-finding test.

11.2.5 Aquatic macrophytes may be exposed to concentrations of formulated chemicals above the reported water solubility of the chemical so it may be informative to test at these concentrations. A true concentration cannot exist above solubility and the term "loading" is used. Testing materials at levels above their water solubility presents several difficulties. At loading levels above solubility, test materials exist in a variety of aggregate forms (for example, particulates, crystals, liquid crystals, etc.). Relatively little is know about the uptake of aggregated compounds into biological membranes and the expression of this toxicity. In fact, toxicity may be due to certain physical effects, such as a reduction in light penetration or interference with nutrient uptake by test material particulates. 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 concentrations up to approximately twice the solubility limit in the nutrient medium. Any observed toxicity above the solubility limit should be clearly identified as such. For test materials of limited aqueous solubility, analytical verification of the solubility under the test conditions can be important.

11.2.6 When the object of a test is to determine the effect of a specific concentration of test material on the growth and development of a test species or whether or not the IC25, IC50, or NOEC value is above or below a specific concentration, only that one concentration (see 13.1) and the controls (see 11.4) are required.

11.2.7 The pH and conductivity of all the concentrations of the test solution should be measured at the beginning of the test. At the end of each test, pH and conductivity of the solution in each test chamber may be measured. Other physical parameters such as water hardness and salinity may also be measured.

11.3 *Test Material Stock Solutions*:

11.3.1 If the test material has a high water solubility, a test chemical stock solution may be made by dissolving the test material in the nutrient solution. For test materials with low water solubility, a solvent can be used to make a stock solution that can then be aseptically added to the nutrient solution.

11.3.2 If a solvent other than the nutrient solution is necessary, its concentration in the test solution should be kept to a minimum and should be low enough that it does not adversely affect either survival or growth of the test plant. Reagent grade or better organic solvents should be used and their concentration in the test solution should not exceed 0.5 mL/L **(48, 49)**. These limitations do not apply to any ingredients of a mixture, formulation, or commercial product unless an extra amount of solvent is used in stock solution preparation.

11.3.3 When a solvent other than the nutrient solution is used, a solvent control must be employed in the test (see 11.4).

11.3.4 If the solvent has an unknown toxicity to the organism, a test using a dilution series of the solvent must be conducted. This will determine if the survival or growth of the test species is affected by the solvent and what concentration of solvent is non-toxic to the test organism. If a solvent test has already been conducted with the same solvent on the same test species using the same reagent water, then the dilution series solvent test does not need to be repeated. Choose another solvent if the solvent test affects the organisms growth or survival. Methanol is non-toxic to *M. sibiricum* up to a concentration of 0.4 % (v/v) **(6)**.

11.3.5 It may be of interest to determine if the chemical and solvent interact at different concentrations. If there is an interaction, this should be taken into account when choosing solvent concentrations **(50, 51)**.

11.3.6 The test material is added to the autoclaved nutrient solution or solvent under sterile conditions (for example, laminar airflow cabinet or UV sterilization hood). It is not necessary to autoclave the test material/nutrient solution or solvent mixture. Liquid test materials may be filter sterilized.

11.4 *Controls*:

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

11.4.2 If a solvent other than water is used, two controls must be included in the test. One control would be the nutrient solution control and the other control would be a nutrient solution/solvent control containing solvent from the same batch used to make the stock solution.

11.4.3 The concentration of solvent in the nutrient solution/solvent control should be equivalent to the concentration used in the test solutions and should be no greater than 0.5 mL/L **(48, 49)**.

11.4.4 The percentage of organisms that show signs of stress, such as necrosis, chlorosis, stem disfigurement, etc., must be 10 % or less for each control type (see Guide E 1841) **(52, 53)**.

11.4.5 If the test contains both a nutrient medium control and a nutrient solution/solvent control, the growth and development of the plants in the two controls should be compared using a t-test or a non-parametric test such as the Mann-Whitney U-test. Another method of comparing the control groups would be to perform an analysis of variance including all treatment and control groups followed by an LSD comparison of the control group means. The test statistic, its significance level, the minimum detectable difference, and the power of the test should be reported.

11.4.6 If a statistically significant difference in growth or development is detected between the two controls, only the solvent control can be used for meeting the requirements of Section 15 and as the basis for calculation of results. If no statistically significant difference is detected, the data from both controls should be used for meeting the requirements of Section 15 and as the basis for the calculations.

11.4.7 Two reference toxicants (positive controls) **(54)** have been tested at this time. Zinc chloride is a more effective reference toxicant than phenol **(6)**. If space and time permit, positive controls may be conducted with each test. Other positive controls may be used after validation in this axenic testing system.

12. Test Organism

12.1 *Recommended Species*—It is recommended that *Myriophyllum sibiricum* Komarov (northern watermilfoil) be used for testing. *M. sibiricum* is ecologically important since it provides food and shelter for other organisms **(55)**. This species is readily available from laboratory sources or it can be easily collected from field sites and sterilized. It is easy to culture and can produce new growth within ten to twelve days. In this test system, asexual reproduction allows the plant to produce numerous experimental plants from a small number of stock plants. Possible sterile sources of stock plants are listed in Appendix X1. This species was previously named *M. exalbescens* Fernald **(13, 55, 56)**.

12.2 *Alternate Species*—Other test species may also be tested following this guide but more research needs to be conducted. Toxicity tests with other species of *Myriophyllum* are currently under development. This bioassay may be modified to screen new chemicals for the control of invasive aquatic plants, such as *Myriophyllum spicatum* (Eurasian watermilfoil).

12.3 *Culturing*:

12.3.1 If starting from field collected, non-sterile plants, collect *M. sibiricum* turions in the autumn. Place the turions into a 20-L aquarium containing 5 cm of sterile sediment that is covered with silica sand or Turface®[5] and 18 L of reagent

water. Aerate the aquarium and maintain at a temperature of 15°C and a fluence rate of 200 to 300 µmol m^{-2} s^{-1} for 16 h per day (see Guide E 1733 for conversion to other fluence rate units). The plant culture in the aquarium may be maintained as a backup source of plants in case the sterile plant cultures are destroyed by mechanical malfunction in the growth cabinet, contamination, or other reason. The plants grown in the aquarium are not sterile and sterile cultures cannot be maintained in a batch culturing system. To sterilize the culture, plants are removed from the aquarium and rinsed under flowing deionized water for about 0.5 h. Under aseptic conditions in a laminar airflow cabinet, the plants are disinfected for 20 min in a 3 % (w/v) sodium hypochlorite solution containing 0.01 % of a suitable surfactant. Agitate the disinfectant and plant material. Segments with several nodes are transferred into sterile culture tubes containing 45 mL of sterilized modified Andrews' medium (see Appendix X2) and capped with plain culture tube closures. Only one plant segment is placed into each test chamber. Laboratory sealant film is used to secure the closure to the culture vessel. Once a sterile culture has been established, plant segments containing several nodes should be transferred to new test chambers containing fresh liquid nutrient media every ten to twelve days. As demonstrated by culturing on agar plates, the plants must be sterile and remain sterile for eight weeks before testing can be initiated.

12.3.2 If starting with a sterile culture, all transfers must be conducted using aseptic techniques. The stock plants are segmented so that each section contains several nodes and visible buds. The test species must be cultured for eight weeks in the new facilities before testing can be initiated. If the plants transferred are going to be utilized for an experiment in ten to twelve days, they should contain only one visible bud no longer than 1 cm. Each segment is placed into a sterile culture tube containing 45 mL of modified Andrew's medium and covered with a sterile plain culture tube closure. Laboratory sealant film is used to secure the closure and the culture tube. Sterile stock plants are maintained by transferring plant segments containing several nodes and visible buds to new test chambers containing fresh liquid nutrient media every ten to twelve days. Only one plant segment is placed in each test chamber. Always leave a few sterile plants untransferred to ensure the continuation of sterile plants in case a batch of freshly transferred plants becomes contaminated.

12.3.3 The stock plant tubes should be alternated in test tube racks (12 by 30 cm with 40 spaces) and placed in an environmental chamber set at 16/8 h photoperiod and a 25/20°C temperature regime. The temperature is lowered during the dark period to simulate natural conditions in temperate climates. Other temperature regimes may be used if it can be demonstrated that they promote healthy *Myriophyllum* growth. The fluence rate at the base of the test tube rack should be 100 to 150 µmol m^{-2} s^{-1}.

12.3.4 Ten to twelve days before each experiment, double the number of plants necessary for the experiment should be transferred. This permits the selection of healthy plants of similar size for the experiment while leaving the additional plants for new stock plant creation. Apical shoots are ready for experimental use when they are at least 3 cm in length.

13. Procedure

13.1 *Experimental Design*—Decisions concerning such aspects of experimental design as the dilution factor, number of treatments, and number of test chambers per treatment should be based on the purpose of the test and the type of result calculations to be performed (see Section 15). One of the following two types of experimental designs will probably be appropriate in most cases.

13.1.1 A growth test intended for the calculation of treatment differences (IC25, IC50, or NOEC) based on a measurable endpoint usually consists of one or more controls and a geometric series of five to seven concentrations of test material. In the nutrient solution control and, if necessary, a nutrient/solvent control (see 11.4), the plants are exposed to nutrient solution to which no test material has been added. Except for the control(s) and the highest concentration of test material, each test concentration should be at least 50 % of the next higher one **(48, 57)**, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5, five to seven properly chosen concentrations are a reasonable compromise between cost and the risk of all concentrations being either too low or too high **(48)**.

13.1.2 If it is only necessary to determine whether a specific concentration unacceptably affects growth and development or whether the IC25, IC50, or NOEC is above or below a certain concentration, only that concentration and the control(s) are necessary. However, two additional concentrations at about one-half and two times the concentration of concern are desirable for increased confidence in the results.

13.1.3 With respect to factors that might affect results within test chambers and the results of the test, all test chambers in the test should be treated similarly. Test chambers are arranged alternately in the test tube racks in up to four rows per rack for a maximum of twenty tubes per rack. Treatments must be randomly assigned to individual spaces and may be randomly reassigned during a test. A randomized block design (with each treatment being present in a block, which may be a row within the test tube racks or a test tube rack) is preferable to a completely randomized design.

13.1.4 A minimum of five replicate test chambers is recommended for use in each treatment of an experiment. Because of the importance of the control(s) in the calculation of results, it might be desirable to use more test chambers for the control(s) than for the other treatments.

13.2 *Temperature*—Tests with *M. sibiricum* should be conducted at 25°C during the light period and 20°C during the dark phase. Temperature should be controlled by placing the test chambers in an environmental chamber. Other temperatures may be used to study the effect of temperature on this species or the effect of temperature on the toxicity of a material to *M. sibiricum*.

13.3 *Illumination*—Light should be provided by either fluorescent or incandescent lights or a mixture of both and provide a fluence rate between 100 to 150 µmol m^{-2} s^{-1} when measured at the base of the test chamber **(6)**. The fluence rate at each

position in the growth cabinet should be measured and should not differ by more than 15 % from the selected fluence rate. Other light intensities and wavelengths, especially that of UV radiation found in sunlight may be used to examine the effect of light on the toxicity of a test material (see Guide E 1733).

13.4 *Beginning the Test*:

13.4.1 A large enough batch of nutrient solution should be prepared so that the desired volume can be placed in each control test chamber, the necessary volume of each test solution can be prepared, evaporative loss during autoclaving is accounted for, and all desired analyses can be performed (see 13.6 and Section 12). Enough test solution of each concentration should be prepared so that the desired volume can be placed in each test chamber and all desired analyses of water quality, test material, etc. can be conducted (see 13.6 and Section 12).

13.4.2 Uniform, healthy-looking plants should be removed from the stock culture for use in testing. Randomly select ten to twelve day old shoots that are approximately 3 cm long. Use plants of the same age and from the same source for each experiment. Aseptically, cut 3–cm apical lengths from the stock plants and transfer them into randomly selected autoclaved test tubes containing 40 mL of sterile test medium. Ensure that the cut end of the apex is touching the sterilized Turface®[5] to optimize rooting. Carefully add a 15–cm measuring rod. The top end of the measuring rod is inserted into the 3.5–cm length of tubing.

13.4.3 The test begins when all the test chambers contain an apical segment. Measure the length of each plant using the measuring rod. Length is measured from the cut end of the plant to the top of the apex.

13.4.4 The tubes should be randomized in alternating holes in test tube racks and placed into a growth cabinet maintained under the conditions outlined in 13.2 and 13.3.

13.4.5 The number of roots and branches produced plus the number of nodes may be measured every second day or less frequently. Every second day, the plant length from the cut stem base to the tip of the apex (mm) may be measured. Exclude any leaves that extend above the apical meristem. The initial height of each plant segment may be subtracted from all subsequent plant height measurements. The plant length data (mm) are used to establish a growth curve and area under the growth curve is calculated by:

$$\text{area under curve} = \sum_{i=2}^{n} \frac{IH_{i-1} + IH_i}{2} \cdot (T_i - T_{i-1}) \quad (1)$$

where: IH_i is the increase in height from the start of the experiment and T is the time at each subsequent measurement point, in hours from time zero **(19, 29)**. An advantage to the test tube system described in this guide is the valuable information obtained from the growth curves. Along with the control growth curve (Curve 1), test materials may produce one of five types of growth curves (see Fig. 2). The test material can have an immediate toxic effect that does not change over time (Curve 2). The test material may not inhibit growth but may or may not affect the other parameters examined (Curve 3). In some cases, the test chemical may appear to be initially toxic but *Myriophyllum* might metabolize the test chemical and the toxic effect is reduced (Curve 4). When there is recovery, the final plant height may not be significantly different from the control plant final height, which emphasizes the importance of measuring plant growth during the 14 days. The test chemical can have a delayed toxic reaction wherein toxicity is not displayed until several days after test initiation (Curve 5). The last scenario is that plant height may be stimulated but there could be an effect on weight or one of the other endpoint parameters (Curve 6). This type of data may be important in

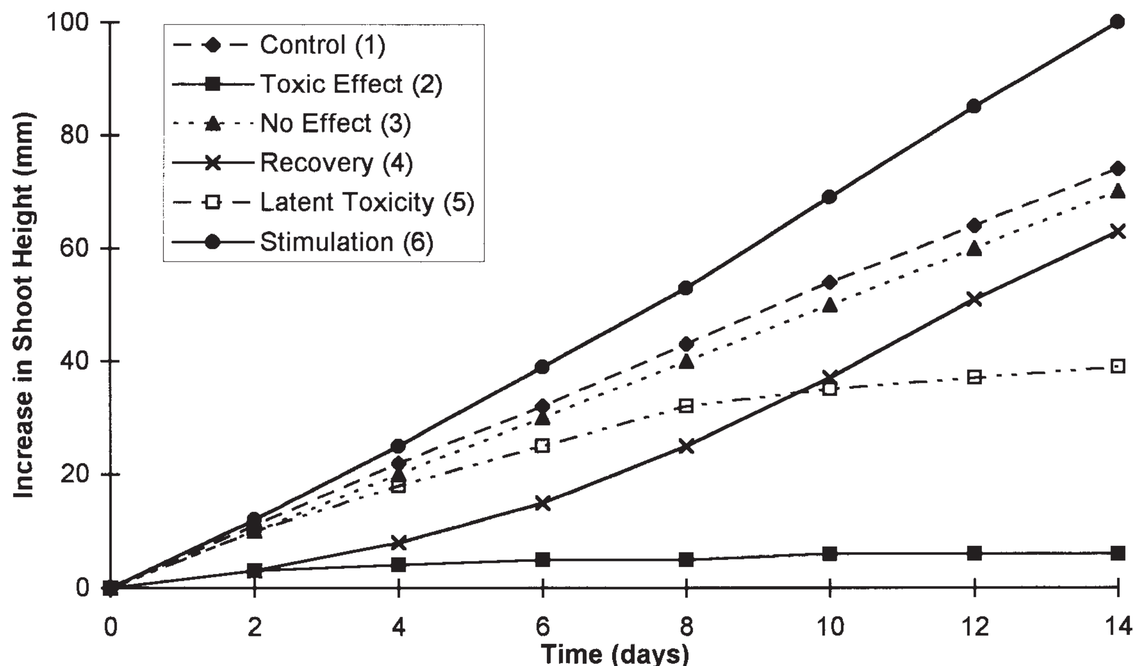


FIG. 2 Hypothetical Growth Curves That may be Obtained During a *Myriophyllum sibiricum* Toxicity Test When the Plants are Exposed for 14 Days to Test Materials with Different Modes of Action

examining chemical metabolism or possible plant recovery from the effects of the test chemical.

13.5 *Duration of Test*—The test ends 14 days after plants are initially placed into the test solutions. A shorter test duration might not be sufficient for toxicity to be demonstrated. A longer duration might allow the plants to adjust to the presence of the test material, produce excess growth that might make enumeration difficult or utilize all the nutrient resources in the medium, thus limiting control growth.

13.6 *Evaluation of Test*:

13.6.1 *Biological Data*—Results of the toxicity tests with *M. sibiricum* should be calculated based on one or more morphological or physiological measurements on the plants in each test chamber **(6)**. The steps listed below are the maximum number of endpoints that have been employed. If after preliminary testing, it is determined that the test chemical has a mode of action that does not affect one of the systems examined, that endpoint can be eliminated. It is recommended that the order of measuring the endpoints be followed but one or numerous endpoints need not be conducted, as determined by the researcher.

13.6.1.1 Visually record the plant length using the mm marks on the Westergren blood sedimentation tube.

13.6.1.2 One at a time, remove the laboratory sealant film and measuring rod from each tube. Measure the D.O. immediately.

13.6.1.3 Remove the plant from the test chamber. Measure the caliper length of the shoot and roots **(26)**, returning the plant to the test chamber to prevent desiccation.

13.6.1.4 Pat each plant dry on paper towels. Measure the fresh weight **(4, 26)**.

13.6.1.5 Using an analytical balance and working quickly, cut off the apex to 50 ± 3 mg. Record the actual fresh weight so that pigment content can be calculated on a weight basis (see 13.6.2.2). Place the apex into a glass scintillation vial containing 10 mL of 80 % ethanol. Store the scintillation vials in a dark cold room (4°C) for 24 h. These apices will be used to determine chlorophyll/carotenoid content.

13.6.1.6 To determine membrane permeability, the next 100 ± 5 mg (fresh weight) of the shoot is triple rinsed in reagent water, placed into a flat bottomed tube containing 20 mL of reagent water, loosely covered and left at room temperature for 24 h. In order to avoid excess cellular leakage, this 100 mg sample should consist of only one section **(58, 59)**.

13.6.1.7 The extra portion of the plant is weighed and dried at 80°C for a minimum of 24 h. Weigh the dried plants.

13.6.2 The 24 h measurements are made 24 h after all the plants are weighed and segmented.

13.6.2.1 *Measurement of Chlorophyll/Carotenoid Content of the Apices*—After the apices have been soaking in 80 % ethanol for 24 h, analyze for pigment content on a spectrophotometer at 470, 647, 663 nm. Calculate values for chlorophyll *a*, chlorophyll *b* and carotenoid content based on either the fresh or dry weight of the apices (see Practice D 3731) **(60, 61)**. Other extraction solvents such as DMSO, acetone and methanol have been used to extract pigments from other plant species **(61-65)** but ethanol has been successfully used to extract chlorophyll *a* from algae and terrestrial plants **(61, 65, 66)**. Ethanol without maceration was efficient at extracting the photosynthetic pigments from *Myriophyllum* **(6)**. As a submersed macrophyte, the leaves of *Myriophyllum* have a very thin cuticle **(14)**. The chloroplasts are abundant throughout the epidermal and mesophyll cells of submersed leaves **(14, 15)** so it is easy to extract the chlorophyll from the apical segments without maceration or other rupturing of the cells. Terrestrial and aquatic emergent leaves have a thick waxy layer and epidermal cells that do not contain chloroplasts **(15)** so the chlorophyll is much harder to extract.

13.6.2.2 *Measurement of Membrane Integrity*—Using a conductivity meter, measure the conductivity of the water/plant solution in the flat bottomed tubes. Boil the flat bottomed tubes for 20 min. Remove the tubes from the water. Allow to cool down to room temperature. Measure the conductivity of the solution again **(58, 59)**. Membrane integrity is determined as percentage of total electrolyte leakage:

$$membrane\ leakage = \frac{conductance\ before\ boiling}{conductance\ after\ boiling} \cdot 100\ \% \qquad (2)$$

14. Analytical Methodology

14.1 The growth medium, stock solutions, or test solutions, or all three may be analyzed for chemical content at the beginning and end of a test. If these samples cannot be analyzed immediately, they should be handled and stored appropriately **(67)** to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, sorption, and volatilization. For example, the solutions may be frozen at –20°C until analysis can be conducted.

14.2 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 **(68)**.

14.3 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 samples are analyzed.

15. Calculation

15.1 Depending on the data to be analyzed and the purpose of the test, a variety of procedures can be used to calculate the results from a test.

15.2 The data may be examined for the presence of outliers and tested for heterogeneity before a randomized complete block analysis of variance (ANOVA) is conducted.

15.3 After the ANOVA, the treatments can be compared to the control using an appropriate mean comparison procedure (for example, Dunnett's). The highest concentration not significantly different from the control is designated the no-observable-effect concentration (NOEC) (see Practice E 1847). The growth rate or the mean percent inhibition actually observed at the NOEC should be calculated.

15.4 If an IC50 is to be determined, first calculate the percent inhibition (% *I*) for each test chamber in each treatment other than the control(s) (see Practice E 1847). Percent inhibition is usually calculated:

$$\% I = \frac{control\ mean - treatment\ value}{control\ mean} \cdot 100\ \% \quad (3)$$

15.4.1 On occasion, it may be necessary to use a modified formula to calculate percent inhibition. This is useful for endpoint parameters, such as membrane integrity, where treatment values increase as toxicity increases:

$$\% I = \frac{control\ mean - treatment\ value}{control\ mean - most\ toxic\ value} \cdot 100\ \% \quad (4)$$

15.4.2 In situations where 100 % inhibition is not equivalent to zero, the following percent inhibition formula can be substituted:

$$\% I = \frac{control\ mean - treatment\ value}{control\ mean - minimum\ value} \cdot 100\ \% \quad (5)$$

15.4.3 The IC50 is then calculated using a regression model. Several statistical programs are available that assist with the analysis of data with a continuous response **(69, 70)**. The type of model and estimation method should be described along with the 95 % confidence intervals about the estimates **(31)**.

15.5 If the test consisted of only one test concentration and the control(s), a %*I* for this concentration may be determined. A t-test or Mann Whitney U-test may be used on the raw or transformed data to determine if the treatment is statistically significantly different from the control(s).

15.6 If the test contains more than one control, such as nutrient solution and nutrient/solvent control, they should be compared and pooled if they are found not to be significantly different (see 11.4). The ANOVA, NOEC, or IC50 procedures, described in 15.2 to 15.5, should be used.

15.7 All endpoints may be useful in risk assessment. Following traditional methods, the endpoint sensitivity may be ranked based upon the IC25, IC50, and NOEC. The mode of phytotoxic action of the test material often determines which endpoint(s) are the most sensitive. The most consistently sensitive endpoint(s) for each test material may be used in environmental risk assessments. Currently, it is difficult to interpret effects observed in the lab in reference to those that could be found in the environment. Since this toxicity test can detect non-lethal physiological endpoints as well as morphological changes, this toxicity test could act as an early warning system for possible environmental effects. If effects are noted in this toxicity test, it could indicate that further lab and field testing may be required. The length of exposure to the toxicant would be an important consideration. If a physiological change is observed in the lab (for example, chlorophyll content), this may be detrimental to a field population with a long term exposure.

16. Acceptability of Test

16.1 A test should be considered unacceptable if one or more of the following occurred:

16.1.1 All test chambers and covers were not identical in size, shape and composition.

16.1.2 Treatments were not randomly assigned individual test chamber locations.

16.1.3 A required nutrient medium or nutrient/solvent control was not included in the test or the solvent concentration affected the growth of the species.

16.1.4 The test organism had not been cultured in the nutrient solution and at the same temperature and fluence rate as used in the test for at least eight weeks prior to the test.

16.1.5 *M. sibiricum* apices were not randomly assigned to test chambers.

16.1.6 The test lasted less than 14 days. It might be possible to present preliminary information if the test duration is less than 14 days.

16.1.7 Temperature and light were not maintained as specified in 13.2 and 13.3.

16.1.8 At the beginning of the test, variation in apical height between test chambers was more than 6 mm.

16.1.9 Ten percent or more of the control organisms demonstrated some form of stress (chlorosis, necrosis, stem disfigurement) **(52, 53)**.

16.1.10 One or more of the test chambers was contaminated with another organism (that is, non-sterile conditions).

17. Report

17.1 Include the following information in the record of the results of an acceptable *M. sibiricum* toxicity test, either directly or by reference to available documents:

17.1.1 Name of test and investigator(s), name and location of the laboratory, and dates and times of initiation and termination of the test, plus dates that the stock plant cultures were initiated,

17.1.2 Source of the test material, its lot and CAS number, composition (identities and concentration of major ingredients and major impurities, if applicable), known chemical and physical properties, and whether it is a commercial product, formulation or active ingredient. The identity and concentration(s) of any solvent used,

17.1.3 Source and chemical characteristics (pH, hardness, conductivity, etc.) of the reagent water plus a description of any pre-use analysis to confirm the absence of pesticides, PCB's, toxic metals, etc.,

17.1.4 The source, composition and lot number of the Turface®[5] used,

17.1.5 Description of the preparation of the nutrient medium,

17.1.6 Source of the test species, scientific name, name of the person who identified the species and the taxonomic key used, and culture procedures used,

17.1.7 Description of experimental design, test chambers and covers, volume of solution in the chambers, and the average apical height at the beginning of the test,

17.1.8 Average and range of the measured temperature and fluence rate, plus the method of measuring both,

17.1.9 Schedule and methods for preparing test solutions,

17.1.10 Methods and results (with standard deviations or confidence limits) of chemical and physical analyses of water quality and test concentration(s). Include validation studies and reagent blanks,

17.1.11 Methods used for measuring the selected endpoints,

17.1.12 A table giving the endpoint data for each test chamber in each treatment including the control(s), in sufficient detail to allow independent statistical analysis,

17.1.13 Definition(s) of the endpoint(s) used for calculating IC50 and NOEC values. A summary of general observations on other effects,

17.1.14 The IC25, IC50 value and 95 % confidence interval, the NOEC value, percent inhibition, and the methods used to calculate them,

17.1.15 The most sensitive endpoint for each test material based upon the IC25, IC50, and NOEC,

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

17.1.17 Any evidence of stimulation found in any treatment,

17.1.18 Anything unusual about the test, any deviation from these procedures, and any other relevant information, and

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

18. Precision and Bias

18.1 The precision and bias for this guide for conducting static, axenic 14-day phytotoxicity tests with the submersed aquatic macrophyte, *Myriophyllum sibiricum* are currently being determined **(6)**.

19. Keywords

19.1 aquatic toxicity testing; *Myriophyllum sibiricum*; phytotoxicity test; submersed aquatic macrophyte

APPENDIXES

(Nonmandatory Information)

X1. SOURCE OF MATERIALS

X1.1 *Source of Myriophyllum sibiricum*[7]:

X1.1.1 In Canada, *M. sibiricum* is currently being cultured by: Department of Environmental Biology, University of Guelph, Guelph, Ontario, CANADA, N1G 2W1.

X1.1.2 In the United States, *M. sibiricum* is being cultured at: Analytical Bio-Chemistry Labs, Inc., 7200 E. ABC Lane, Columbia, Missouri, 65202, USA.

[7] These suppliers have been found satisfactory for the purposes outlined in this axenic toxicity test but ASTM does not endorse them.

X2. NUTRIENT SOLUTION

X2.1 Modified Andrews' medium **(5, 29)** for experimental toxicity tests with *Myriophyllum sibiricum*, is given in Table X2.1.

X2.2 Stock nutrient solutions are made by dissolving the above salts into reagent water. Solution 11 (FeEDTA) is made by dissolving 372 mg Na_2EDTA in 1000 mL reagent water. Once this is dissolved, add 278 mg $FeSO_4 \cdot 7H_2O$ and heat to approximately 80°C **(5)**. These stock nutrient solutions may be stored in the dark at 4°C **(31)** for a maximum of six months. The cold storage room must be free from volatile compounds.

X2.3 The liquid culture medium is prepared by adding the appropriate volume of each stock nutrient solution to 2 L of sterile reagent water. Into each 2 L of liquid nutrient medium, 60 g of sucrose is added **(71)**. Adjust the pH to 5.8 ± 0.1 with 1N KOH or HCl.

TABLE X2.1 Modified Andrews' Medium (5, 29) for Experimental Toxicity Tests with *Myriophyllum sibiricum*

Solution Number	Salt	Weight of salt per 1 L stock solution	mL stock solution per 2 L final volume
1	KNO_3	16.16 g	10.0
2	$Ca(NO_3)_2 \cdot 4H_2O$	37.76 g	10.0
3	$MgSO_4 \cdot 7H_2O$	19.72 g	10.0
4	KH_2PO_4	5.44 g	10.0
5	KCl	746 mg	2.0
6	H_3BO_3	155 mg	2.0
7	$MnSO_4 \cdot H_2O$	169 mg	2.0
8	$ZnSO_4 \cdot 7H_2O$	115 mg	2.0
9	$CuSO_4 \cdot 5H_2O$	12.5 mg	2.0
10	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	3.7 mg	2.0
11	FeEDTA	372 mg Na_2EDTA 278 mg $FeSO_4 \cdot 7H_2O$ heat to 80°C	20.0

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