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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]

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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 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 because there is no degradation of the test item by micro-organisms. For similar reasons, other aquatic plant testing, such as those of, *Lemna* and algae, is commonly conducted in an axenic fashion. Overall environmental impact can not be directly determined. The main other disadvantage of the axenic test system is the difficulty in preventing accidental contamination. These procedures could possibly be useful for conducting toxicity tests with other species of submersed macrophytes, although modifications might be necessary (**1-8**)[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 E1192). 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 E1415), 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 D3731 and Guide E1218) (**9-18**). 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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[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:*[3]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

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

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

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

E1218 Guide for Conducting Static Toxicity Tests with Microalgae

E1415 Guide for Conducting Static Toxicity Tests With *Lemna gibba* G3

E1598 Practice for Conducting Early Seedling Growth Tests (Withdrawn 2003)[4]

E1733 Guide for Use of Lighting in Laboratory Testing

E1841 Guide for Conducting Renewal Phytotoxicity Tests With Freshwater Emergent Macrophytes

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

3. Terminology

3.1 *Definitions:*

3.1.1 The words *must, should, may, can,* and *might* have very specific meanings in this guide. *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 D1129, Guide E729, Terminology E943, and Practices E1598 and E1847.

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 **(19-23).**

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

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

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

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growth and development of plants in an appropriate control. A test substance is considered biologically active when a statistically significant concentration-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 chemical. In this axenic testing system, 3 g of Turface[5] has proven successful as an artificial rooting medium (**9**). 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, oxygen evolution, and chlorophyll fluorescence.

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 rooted vascular plants (**25-28**).

5.2 Toxicological research with terrestrial and aquatic vascular plants has been initiated (**29**) including the development of a protocol for testing with emergent macrophytes (Guide E1841) (**30**). However, protocols for phytotoxicity testing with freshwater submersed plants still require development. Toxicological research has been conducted using submersed macrophytes (**1-8, 31-35**) 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 (**36**). Lower temperatures in autumn initiate the formation of turions on lateral branches that develop into new plants when environmental conditions become favorable (**19-23**). 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 (**9-15**).

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 D3978, **37-39**), floating aquatic macrophytes (Guide E1415, **37, 40**) 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)) (**25-27**). 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 (**41**). 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, 36**). Furthermore, test tubes permit height measurements *in situ* (**36**).

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 may be 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 (**31-35**) and chlorophyll fluorescence (**42**) might be other endpoints that could be explored. Some endpoints have been compared (**43, 44**), but selected appropriate endpoint(s) based upon mode of action or route of exposure.

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 E1023). Plants cultured using this method have also been used to determine the effects of toxicants on plants in microcosms (**45**).

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. Therefore, this

[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 (**24**), may reduce light penetration by adhering to the test tube or plant or by remaining suspended in the nuntrient medium (**9**).

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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 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 (**40**). 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 E1733. 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) or non-toxic foam plug. 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 (**24**) are not as effective for culturing *M. sibiricum* in this sterile test system (**9**). 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. The test chambers may also be covered with non-toxic foam plugs.

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 for 20 min, at 121°C and $1.31 \cdot 10^5$ Pa. The liquid cycle should contain a slow exhaust portion. Guide E1218 recommends microwaving as an acceptable alternative to autoclaving and this technique has been used for the sterilization of soil (**46**) and phytoplankton culture medium (**47**). Microwaving times and temperature cycles should be investigated before being used in this test method because microwaving might not acceptably eliminate the microbial population (**48**).

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 or a biological safety cabinet is 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.*

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