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Standard Guide for Conducting Sexual Reproduction Tests with Seaweeds[1]

This standard is issued under the fixed designation E1498; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers procedures for obtaining laboratory data concerning the adverse effects of a test material added to dilution water on sexual reproduction by seaweeds. The exposure duration is species dependent and is followed by a period of development to allow the evidence of sexual reproduction to appear. There is no exposure to toxicants during the development period. This restricts the tests primarily to the events surrounding egg fertilization, and it minimizes any timelag effects on development that might interfere with correct enumeration of the number of sexual events that occurred. These procedures will probably be useful for conducting sexual reproduction toxicity tests with a variety of species of seaweeds, although modifications might be necessary.

1.2 Other modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, the results of tests conducted using unusual procedures are not likely to be comparable to those of many other tests. Comparison of the results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting sexual reproduction tests with seaweeds.

1.3 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, and known mixtures or whole effluents, as well as for use in testing surface waters. With appropriate modifications, these procedures can be used to study the effects of temperature, dissolved oxygen, pH, and such materials as leachates, oils, particulate matter, and sediments.

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

1.5 This guide is arranged as follows:

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1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazards statements, see 6.4 and Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System) (Withdrawn 1997)[3]

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

[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Actionand is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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[2] 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.

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

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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

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 condition, unless the purpose of the test requires a different design. "Must" is used only in connection with factors that relate directly to the acceptability of the test (see Section 12). "Should" is used to state that the specified condition is recommended and ought to be met, if possible. Although the violation of one "should" is rarely a serious matter, the 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." Therefore, the classic distinction between may and can is preserved, and might is never used as a synonym for either "may" or "can."

3.1.2 For definitions of other terms used in this guide, refer to Guide E729, Terminology E943, and Guide E1023. For an explanation of units and symbols, refer to Practice E380.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *cystocarp*—a structure produced by the female red algal gametophyte in response to fertilization.

3.2.2 *gametophyte*—the sexual, gamete-producing phase in the life history of a plant.

3.2.3 *ostiole*—an opening.

3.2.4 *sorus (plural sori)*—a group or cluster of reproductive structures, for example, spermatangia, producing male gametes.

3.2.5 *spermatia*—male gametes in red algae; non-motile and colorless.

3.2.6 *trichogyne*—an elongation of a female oogonium (egg cell) to which male gametes become attached.

4. Summary of Guide

4.1 In each of two or more treatments, female and male gametophytes are exposed in each of three or more test chambers for two days under static or renewal conditions. In each of one or more control treatments, the gametophytes are maintained in dilution water to which no test material has been added, in order to provide the following: (*1*) a measure of the acceptability of the test by giving an indication of the quality of the plants and suitability of the dilution water, test conditions, and handling procedures; and (*2*) the basis for interpreting data obtained from the other treatments. In each of one or more treatments, the gametophytes are maintained in dilution water to which a selected concentration of test material has been added. At the end of the exposure period, female gametophytes are removed and incubated (if necessary) for an additional period of time in toxicant-free medium to allow the development of structures created from sexual reproduction (that is, germination of the zygote). At the end of the development period, sexually produced structures are counted, and the number in the controls is compared with those in the treatments to determine the effect of the test material. The results can be reported as either IC50 or No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) based on sexual reproduction.

5. Significance and Use

5.1 Seaweeds have historically been considered less useful for toxicity testing than microalgae (**1**),[4] and microalgae are often considered less sensitive than aquatic animals (**2**). Such conclusions concerning seaweed insensitivity were based on data for only a few hardy species and based generally on vegetative growth of adult stages as the primary endpoint. The sensitivity of seaweeds increases when effects on sexual reproduction are assessed. This has been shown for *Champia parvula*(**3**), as well as for the brown seaweeds, *Fucus edentatus, Laminaria saccharina*, and *Macrocystis pyrifera* (**4**).

5.2 The results of sexual reproduction tests with seaweeds might be useful for predicting the long-term effects likely to occur on seaweeds in field situations due to exposure under comparable conditions.

5.3 The results of sexual reproduction tests with seaweeds might be used to compare the chronic toxicities of different materials, and also to study the effects of various environmental factors on the results of such tests.

5.4 The results of sexual reproduction tests with seaweeds might be an important consideration when assessing the hazards of materials to aquatic organisms or when deriving water quality criteria for saltwater organisms (**5**).

5.5 The results of sexual reproduction tests with seaweeds will depend on the temperature, composition of dilution water, condition of test organisms, and other factors such as light and media.

6. Apparatus

6.1 *Facilities*—Stock cultures and test chambers should be maintained in constant-temperature incubators or water baths. The air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Filtration of the air through a 0.22-µm bacterial filter might be desirable to help prevent the contamination of stock cultures with air-borne microalgae. To further reduce the possibility of contamination by test materials and other substances, especially volatile ones, the stock cultures ought not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or

[4] The **boldface** numbers in parentheses refer to the list of references at the end of this guide.

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equipment is cleaned. A timing device should be used to provide a 16-h light and 8-h dark photoperiod.

6.2 *Construction Materials*—Equipment and facilities that contact the stock solutions, test solutions, or any water into which the test organisms will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that affect the growth or reproduction of seaweeds adversely. In addition, equipment and facilities that contact the stock solutions or test solutions should be chosen to minimize the sorption of test materials from water. Glass is preferred and should be used whenever possible, although some brands of polycarbonate and polystyrene have been used with success. Brass, copper, lead, galvanized metal, and natural rubber should not contact dilution water, stock solutions, or test solutions before or during the test. Items made of neoprene rubber, or other materials not mentioned previously, should not be used unless it has been shown that their use will not affect the survival, growth, or reproduction of seaweeds adversely.

6.3 *Test Chambers:*

6.3.1 In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections. The chambers should be covered to keep out extraneous contaminants and to reduce the evaporation of test solution and test material. The cover should be transparent to minimize shading. All chambers, covers, and compartments in a test should be identical.

6.3.2 The volume used for exposure of the test organisms is species dependent. The use of excessively large volumes of solution in the test chambers will probably increase unnecessarily the amount of dilution water and test material used. Glass, 125-mL Erlenmeyer flasks, and 200-mL polystyrene party cups have been used successfully.

6.4 *Cleaning*—All culture glassware should be acid-stripped in 15 % HCl after being washed with a phosphate-free detergent by soaking in 15 % concentrated HCl for at least 10 min (or with three successive rinses with dilute acid coating the entire interior surface). Following the acid treatment, rinse three times in deionized water. The acid treatment is necessary because some detergents can leave a residue that is toxic to seaweeds. Culture glassware should be baked periodically (at least every six months) in a muffle furnace to remove the organic material that might build up on its surface. Alternately, a few mL of concentrated sulfuric acid can be rolled around the inside of wet glassware. **Caution:** The addition of acid to the wet glassware generates heat.

6.4.1 At the end of a test, all items that will be used again should immediately be (*1*) emptied; (*2*) rinsed with water (tap water is permissible at this stage); (*3*) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic chemicals); and (*4*) cleaned as above for culture glassware.

6.5 *Acceptability*—Before sexual reproduction tests with seaweeds are conducted in a new test facility or with new equipment, it is desirable to conduct a nontoxicant test, in which all test chambers contain dilution water with no added test material. This will determine the following before the first test: (*1*) whether the species selected to be used reproduces acceptably; (*2*) whether the water and culture and handling procedures are acceptable; (*3*) whether there are any location effects on reproduction; and (*4*) the magnitude of the within chamber and between-chamber variances.

7. Hazards

7.1 Many materials can affect humans adversely if the precautions taken are inadequate. Skin contact with all test materials and solutions should therefore be minimized by wearing appropriate protective gloves (especially when washing equipment or putting hands into test solution), laboratory coats, aprons, and glasses, and by using forceps to remove seaweeds from test solutions. Special precautions, such as covering the test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**6**), recommended handling procedures (**7**), and chemical and physical properties of the test materials should be studied before a test is begun. Special procedures might be necessary with radiolabeled materials (**8**) and with test materials that are, or are suspected of being, carcinogenic (**9**).

7.2 Although the 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. The removal or degradation of test material might be desirable before the disposal of stock and test solutions.

7.3 The cleaning of equipment with a volatile solvent, such as acetone, should be performed under a fume hood within a room in which no smoking is allowed and no open flame, such as a pilot light, is present.

7.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

7.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

7.6 Because salt water is a good conductor of electricity, the use of ground fault systems and leak detectors is recommended strongly in order to help prevent electrical shocks.

8. Dilution and Culture Water

8.1 *Requirements*—The water used for seaweeds should (*1*) be in adequate supply, (*2*) be acceptable to the species to be used, (*3*) be of uniform quality, and (*4*) not unnecessarily affect the results of the test.

8.1.1 The dilution water must allow satisfactory reproduction of the species being tested.

8.1.2 The quality of the dilution water should be uniform during the test. The difference between the highest and lowest salinities measured during the test should be less than 2 g/kg. Each pH measured should be between 7.8 and 8.2.

8.1.3 The dilution water should not affect the results of reproduction by seaweeds unnecessarily because of the findings

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TABLE 1 Recipe for Nutrients to be Added for Stock Cultures and for Toxicity Test Medium[A]

Compound	Culture Medium	Test Medium
Deionized water	to 1000 mL	to 1000 mL
Sodium nitrate ($NaNO_3$)	6.35 g	1.58 g
Sodium phosphate ($NaH_2PO_4 \cdot H_2O$)	0.64 g	0.16 g
$Na_2EDTA \cdot 2H_2O$)	133 mg	(2.5 mg)[B]
Sodium citrate ($Na_3C_6H_5O_7$)	51 mg	12.8 mg
Iron[C]	10 mL	2.5 mL
Vitamins[C]	10 mL	2.5 mL
Trace Elements[B,C]	10 mL	...

[A] Add 10 mL/L of culture or test medium.
[B] Use only if 100 % artificial seawater is used.
[C] See Table 2.

as sorption or complexation of test material. Therefore, concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L (Guide E729).

8.1.4 If it is desirable to study the effect of an environmental factor such as TOC or particulate matter on the results of a sexual reproduction test with seaweeds, it will be necessary to use water naturally or artifically high in TOC or particulate matter. If such water is used, it is important that analyses be performed adequate to characterize the water and that a comparable test be available or be conducted in a more usual dilution water to facilitate interpretation of the results in the special water.

8.2 *Source and Treatment*—The best culture medium for most seaweeds is clean, enriched, natural seawater. Some commercial, artificial seawater preparations may support good growth of seaweeds as long as enough of a chelator, such as ethylenediaminetetraacetic acid (EDTA), is present in the nutrient medium. However, chelators need to be reduced or eliminated if heavy metals are being tested. In addition, although some seaweeds can grow well in these artificial seawaters, they may not exhibit their correct morphology and often will not reproduce well, if at all.

8.2.1 The seawater for seaweed cultures is filtered at least to 0.45 µm to remove particulates and then autoclaved for 30 min at 15 psi. If a precipitate forms, filter the seawater through a sterile 0.22 or 0.45-µm membrane filter before used. If natural seawater is used, it should be obtained from an uncontaminated, uniform, quality source. If surface water is used, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination (for example, suspended approximately 1 m below a float). If the source of seawater is of poor quality, treating it with activated carbon before autoclaving may enhance its quality to an acceptable level (**10**).

8.2.2 Several artificial seawater (ASW) recipes made from off-the-shelf reagent-grade salts[5] have also been tried for seaweed growth and reproduction. A modified GP2 (**11**) has been used with limited success (Table 1), although 100 % GP2 does not yield good results with all species consistently. Artificial seawater is autoclaved in the same manner as natural seawater. The use of artificial seawater for cultures for those species that will not grow well in artificial seawater should be restricted to mixing it 50-50 with natural seawater. This mixture can provide a more seasonally stable medium than does natural seawater alone. Although attempts to culture some seaweeds in artificial seawater have not been completely successful, 100 % ASW has been used for an exposure medium.

8.2.3 The original recipe for GP2 (and many other artificial seawaters) requires that the hydrated and anhydrous salts be dissolved in separate solutions and then combined after autoclaving and cooling to avoid precipitation. If sodium bicarbonate is omitted from the medium, however, all other salts can be dissolved in the same solution and autoclaved without precipitation. Preweighed sodium bicarbonate is autoclaved dry in a separate container and added to the salt solution after autoclaving and cooling. Alternately, the dry bicarbonate can be autoclaved in a polytetrafluoroethylene squeeze bottle and then dissolved with sterile, deionized water. The bicarbonate is then added directly to the medium just prior to use.

9. Test Material

9.1 *Single Chemicals*—Single chemicals should be reagent grade or better, unless a test on a formulation, commercial product, or technical grade or use grade material is specifically needed. Before a test is begun, the following should be known concerning the test material:

9.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than approximately 1 % of the material.

9.1.2 Solubility and stability in the dilution water.

9.1.3 Measured or estimated toxicity to the test species.

9.1.4 Precision and bias of the analytical method at the planned concentrations of the test material, if the concentration is to be measured.

9.1.5 Estimate of toxicity to humans.

9.1.6 Recommended handling procedures.

9.2 *Stock Solutions*—The test material can be added directly to the dilution water in some cases, but it is usually dissolved in a solvent to form a stock solution that is then added to the dilution water. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. If the test material is subject to photolysis, the stock solution should be shielded from light.

9.2.1 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is dilution water, although filtration or sterilization, or both, of the water might be necessary. Deionized or distilled water may be used if the salinity of the dilution water will not be affected. Several techniques have been developed specifically for preparing aqueous stock solutions of slightly soluble materials (**12**). The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous solution, but such reagents might affect the pH of test solutions appreciably. The use of a more soluble form of the test material, such as a chloride or sulfate salt of organic amines, sodium or potassium

[5] All reagents should conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. See *Analar Standards for Laboratory U.K. Chemicals*, BDH Ltd., Poole, Dorset, and the *United States Pharmacopeia*.

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