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

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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:

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:*

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[2]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[2]

E 943 Terminology Relating to Biological Effects and Environmental Fate[3]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]

E 1192 Guide for Conducting Acute Toxicity Test on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians[3]

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.01on Aquatic Assessment and Toxicology.

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[2] *Annual Book of ASTM Standards*, Vol 14.02.

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

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 E 729, Terminology E 943, and Guide E 1023. For an explanation of units and symbols, refer to Practice E 380.

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 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

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

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 such things 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 E 729).

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.

[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."

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.

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 salt of phenols and organic acids, and chloride or nitrate salt of metals, might affect the pH more than the use of the necessary minimum amount of a strong acid or base.

9.2.2 If a solvent other than dilution water is used, its concentration in the test solutions should be kept to a minimum and should be low enough that it does not affect the test species. Triethylene glycol is often a good organic solvent for preparing stock solutions because of its low toxicity and volatility and strong ability to dissolve many organic chemicals. Other water-miscible organic solvents such as methanol, ethanol, and acetone may also be used, but they might stimulate the undesirable growth of microorganisms, and acetone is volatile. If an organic solvent is used, it should be reagent-grade or better, and its concentration in any test solution should not exceed 0.5 mL/L. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions. (These limitations do not apply to any ingredient in a mixture, formulation, or commercial product unless an extra amount of solvent is used in preparation of the stock solution.)

Fresh stock solutions should be prepared before each test to minimize any changes in the toxicant due to degradation.

9.2.3 If a solvent other than water is used, at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test, and a dilution-water control should be included in the test. If no solvent other than water is used, a dilution-water control must be included in the test.

9.2.4 If the concentration of solvent is the same in all of the test solutions containing test material, the solvent control must contain the same concentration of solvent. If the solvent concentration is not the same in all of the test solutions containing test material, either (*1*) a solvent test must be conducted to determine whether reproduction by a test species is related to the solvent concentration over the range used in the toxicity test or (*2*) such a solvent test must have already been conducted using the same dilution water. If reproduction is found to be related to solvent concentration, a toxicity test with that water is unacceptable if any treatment contained a solvent concentration in that range. If reproduction is not found to be related to solvent concentration, a toxicity test with that water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

9.2.5 If the test contains both a dilution-water control and a solvent control, the reproduction by the species being tested in the two controls should be compared (see Section 14). If a statistically significant difference in reproduction is detected between the two controls, only the solvent control may be used for meeting the requirements of test acceptability and as the basis for calculation of the results. The data from both controls should be used if no statistically significant difference is detected.

9.2.6 If a solvent other than water is used to prepare a stock solution, it might be desirable to conduct simultaneous tests on the test material using two chemically unrelated solvents or two different concentrations of the same solvent in order to obtain information concerning the possible effects of solvent on the test results.

9.3 *Effluents and Surface Waters*—Effluents can be treated as above for single chemicals at concentrations below or equal to 10 %. For concentrations above 10 %, and for surface waters with a salinity below 28 g/kg, the salinity is adjusted to 30 g/kg with either hypersaline brine (should not exceed 100 g/kg) made by evaporating (heating at 40 to 60°C) or freezing natural seawater, or by making 3 × artificial seawater. Specific information regarding the collection and handling of effluents and surface waters can be found elsewhere (Ref **(12)** and Guide E 1192).

9.4 *Test Concentrations*:

9.4.1 If the test is intended to permit the calculation of an IC50 (or other point estimate), test concentrations should bracket the predicted effect concentration. The prediction might be based on the results of a test on the same, or similar, material with the same, or a similar, species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test, in which groups of organisms are exposed to a control and three to five concentrations of the test material that differ by a factor of ten. The endpoint for range-finding can be death or necrosis instead of sexual reproduction. However, the greater the similarity between the range-finding test and the actual test, the more useful the range-finding test will be.

9.4.2 Concentrations of single chemicals above solubility should be used, if necessary, because organisms in the real world are sometimes exposed to concentrations above solubility and solubility in dilution water is often not well known. The use of concentrations more than ten times greater than solubility is probably not worthwhile. With some test materials, it might be found that concentrations above solubility do not affect sexual reproduction to a greater extent than for test organisms exposed at the solubility limit; such information is certainly worth knowing.

9.4.3 In some (usually regulatory) situations, it is necessary to determine only whether a specific concentration of test material is toxic to the test species. For example, the specific concentration might be the concentration occurring in a surface water, that resulting from direct application of the material to a body of water, or the solubility limit of the material in water. When interest exists only in a specific concentration, it is often necessary to test only that concentration, and it is not necessary to determine a point estimate such as IC50.

9.4.4 If the test is intended to provide a good estimate of the highest concentration of test material that will not affect the sexual reproduction of seaweeds unacceptably, then the test concentration should bracket the best prediction of that concentration.

10. Test Organisms

10.1 *Species*—Whenever possible and appropriate, tests should be conducted with species that have been used successfully in sexual reproduction toxicity tests. Use of the species listed in the appendix is encouraged in order to increase the comparability of results and the availability of much information on a few species rather than a little information on many species. The use of a specific strain should be specified only when it is of special concern. The species used should be determined using an appropriate taxonomic key.

10.2 *Life Stage*—Age is not a factor in the selection of test organisms since seaweeds exhibit indeterminate growth. Life stage, however, is important. Because the test endpoint is sexual reproduction, gametophytes are the life stage needed.

10.3 *Source*—All organisms in a test must be from the same source. When available or possible, laboratory cultures will provide at any time of the year gametophytes whose history and quality are known. If wild populations of seaweeds are used, collections should be obtained from relatively unpolluted areas.

10.4 *Culture Nutrient Medium*—If laboratory cultures are to be used, several precautions are desirable to maximize the efficiency of growing seaweeds. To guard against microalgal contamination, a sterile technique should be used. This includes autoclaving all stock solutions and flaming all tools before cutting or transferring the plants. The culture flasks are capped with aluminum foil and autoclaved dry for 10 min. Axenic (bacteria-free) cultures are not necessary, but uni-algal cultures will prevent competition for light and nutrients by other algae species.

10.4.1 The culture medium is formed by dispensing seawater into the sterile flasks and adding the appropriate nutrients from a sterile stock solution. Many nutrient recipes have been used successfully for laboratory cultures of seaweeds. Success has been achieved with the nutrient recipe listed in Table 2 (which is based on the nutrients for GP2 **(11)**), Medium *f* **(14)**, Provasoli's medium **(15)**, and the commercially available AlgoGro.[6] Healthy, actively growing and reproducing plants are the goal; a standard nutrient recipe for culture is not as critical as a standard nutrient recipe for the exposure medium. However, plants should be cultured in the same dilution source water as that used in tests to minimize the need for acclimating the seaweeds prior to use in tests.

10.4.2 It is convenient to make separate super stock solutions of both the trace elements and vitamins (Table 3). This will greatly accelerate the process of making new complete nutrient stock solutions. The vitamin solution is made and partitioned into 10-mL aliquots in test tubes. These are autoclaved very briefly (1 to 2 min after reaching standard temperature and pressure) and then stored refrigerated. Tubes of vitamins can also be stored frozen and then thawed prior to use. Vitamins are added to the nutrient medium after it is autoclaved. Trace elements are added to the nutrient medium before it is autoclaved. The nutrient medium is adjusted to pH 2.0 with HCl prior to autoclaving in order to prevent precipitation. Since only 10 mL of the nutrient medium is added per liter of seawater, the natural buffering capacity of the seawater is sufficient to keep the pH of the stock culture stable. A pH value between 7.8 and 8.2 is usually best.

11. Procedure

11.1 *Preparation of Plants for a Test*—How gametophytes are prepared or preconditioned, if necessary, for a test is species dependent.

11.2 *Test Conditions*—The specific test conditions for light, salinity, temperature, and nutrient will be species specific.

[6] Available from Carolina Biological Supply, Burlington, NC 27215.

TABLE 2 Super Stock Solutions for Trace Elements, Vitamins, and Iron

Compound[A]		Amount per 500 mL
Trace Elements		
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	121 mg
Potassium iodide	KI	415 mg
Zinc sulfate	$ZnSO_4 7H_2O$	109 mg
Sodium vanadate	Na_3VO_4	30.5 mg
Manganous chloride	$MnCl_2 \cdot 4H_2O$	7.0 mg
Vitamins[B]		
ThiaminùHCl		4.88 g
Biotin		2.5 mg
B12		2.5 mg
Iron[C]		
Ferric chloride	$FeCl_3 \cdot 6H_2O$	2.42 g

[A] Separate stock solutions are prepared.
[B] Adjust to approximately pH 4 before autoclaving for 2 min.
[C] 1 mg Fe/mL.

TABLE 3 Recipe for GP2 Artificial Seawater

Compound		g/L[A]
Sodium chloride	NaCl	21.03
Sodium sulfate	Na_2SO_4	3.52
Potassium chloride	KCl	0.61
Potassium bromide	KBr	0.088
Sodium borate	$Na_2B_4O7 \cdot 10H_2O$	0.034
Magnesium chloride	$MgCl_2 \cdot 6H_2O$	9.50
Calcium chloride	$CaCl_2 \cdot 2H_2O$	1.32
Strontium chloride	$SrCl \cdot 6H_2O$	0.02
Sodium bicarbonate[B]	$NaHCO_3$	0.17

[A] Reduced to yield approximately 30 g/kg salinity; generally made in 10 to 20 L batches.
[B] A stock solution of 68 mg sodium bicarbonate/mL is prepared by autoclaving it as a dry powder and then dissolving it in sterile deionized water. For each L of GP2, use 2.5 mL of this stock solution.

11.3 *Experimental Design*—Decisions concerning aspects of experimental design such as the dilution factor, number of treatments, number of test chambers, and number of gametophytes per treatment should be based on the purpose of the test, and they will be species specific to a large extent. Other standards, such as Guide E 729, are useful in determining the experimental design for seaweed sexual reproduction, even though they were written for aquatic animals.

12. Acceptability of Test

12.1 Acceptability of the test results will depend partly on which species is being tested.

12.2 A toxicity test should usually be considered unacceptable if one or more of the following occurred:

12.2.1 All test chambers were not identical;

12.2.2 Treatments were not assigned randomly to individual test chambers (if the test organisms are in the chambers prior to toxicant), or test organisms were not assigned randomly to treatments (if toxicant is added before the organisms);

12.2.3 A dilution-water or solvent (if required) control was not included in the test;

12.2.4 The test organisms were not cultured in the same water as that used for dilution at least 48 h prior to test initiation;

12.2.5 Sexual reproduction results for the control did not meet species-specific requirements; and

12.2.6 All replicates from the effect concentration chambers should show an effect.

12.3 The test results should usually be considered unacceptable if either of the following occurred (unless the experimental design is to determine whether there is an effect at a single concentration):

12.3.1 All treatments other than control treatments caused significant effects on sexual reproduction; and

12.3.2 No treatments caused significant effects on sexual reproduction.

13. Calculation of Results

13.1 The primary data to be analyzed from a sexual reproduction test with seaweeds are those on (*1*) the number of sexual products produced and (*2*) the concentration of test material in the test solutions in each treatment (if such concentrations were measured).

13.2 The variety of procedures that can be used to calculate the results of sexual reproduction tests with seaweeds can be divided into two categories: (*1*) those that test hypotheses and (*2*) those that provide point estimates. No procedure should be used without careful consideration of the advantages and disadvantages of various alternative procedures and appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of results should be appropriate to the experimental design.

13.2.1 It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test material affected a statistically significant difference in sexual reproduction from the control treatment(s). If a hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures and heterogeneity tests. Then, a pairwise comparison technique, analysis of variance, or multiple comparison procedure appropriate to the experimental design should be used. Presentation of the results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test.

13.2.2 A point estimate, with its corresponding confidence interval, is often desirable. In this case, an inhibition concentration (for example, IC50) that results in a specific level of reduction relative to the control response is calculated. Because the control response in seaweed sexual reproduction data is variable, some of the traditional methods for calculating point estimates for mortality data with animals do not apply. A linear interpolation method **(16)** has been proposed for use in these situations and has been used for seaweed sexual reproduction data **(17)**.

14. Documentation

14.1 Include the following information, either directly or by reference to available documents, in the record of results of an acceptable reproductive test with seaweeds:

14.1.1 Names of the test and investigator(s), name and location of the laboratory, and dates of initiation and termination of the test.

14.1.2 Source of the test material. If a single chemical, its lot number, composition (the identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used. If an effluent, the source and date and method of collection, and the method of shipment and storage prior to testing.

14.1.3 Source of the dilution seawater and description of any pretreatment.

14.1.4 Source of the seaweed, name of the person who identified the plant, acclimation and culture procedures used, and observed condition of the gametophytes prior to the test.

14.1.5 Description of the experimental design, test chambers, and volume of solution used in the chambers. Description of the lighting and nutrient medium.

14.1.6 Range and average measured test salinity and temperature, and the method used to measure both.

14.1.7 Schedule for obtaining samples of the test solutions, and methods used to obtain, prepare, and store them.

14.1.8 Methods used for, and results of (with standard deviations or confidence limits) any chemical analyses of water quality and concentration(s) of test material, impurities, and reaction and degradation products, including validation studies and reagent blanks.

14.1.9 A table of data on the number of cystocarps per plant in each test chamber for each treatment, including the control(s), in detail sufficient to allow independent statistical analysis.

14.1.10 Methods used for, and results of, statistical analyses of the data.

14.1.11 Summary of general observations on other effects.

14.1.12 Results of all associated reference toxicant tests.

14.1.13 Anything unusual concerning the test, any deviation from these procedures, and any other relevant information.

14.2 Include enough information in published reports to identify the procedures used and quality of the results clearly.

15. Keywords

15.1 algae; *Champia*; microalgae; plants; seaweed; toxicity test

APPENDIX

(Nonmandatory Information)

X1. *CHAMPIA PARVULA* (RHODOPHYTA)

X1.1 Plants of the red seaweed, *Champia parvula* (C. Agardh) Harvey, are bushy and 5 to 10-cm tall in the field. The main axis and branches are cylindrical, hollow, and septate. *Champia's* life history is an alternation of isomorphic generations (Fig. X1.1). Although three macroscopic stages exist in the life history, only the male and female plants are used in toxicity testing. *Champia* is distributed widely throughout the world. On the east coast of North America, it extends from Cape Cod down through the Caribbean and into the Gulf of Mexico. On the west coast, it extends from southern California down into Mexico. The clone currently in wide use was isolated from Rhode Island water in 1979. It is probably not essential to have a standard clone, but some experience and time is required to isolate new clones from nature into unialgal culture. Unialgal stock cultures are necessary to maintain healthy, actively growing plants for use in testing.

X1.2 Male and female plants of *Champia* can be maintained easily in unialgal culture in the laboratory. New cultures can be started from excised branches, making it possible to

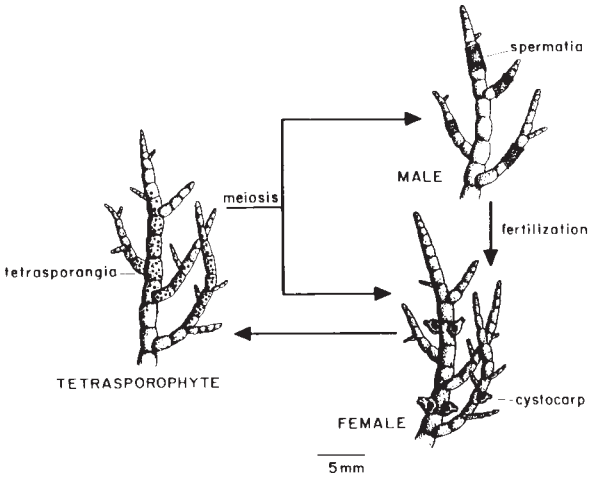

FIG. X1.1 Life History of *Champia parvula*

maintain clonal material indefinitely. No special preconditioning is required to induce reproduction. Under the conditions listed below, male gametophytes produce spermatia continuously, and females are always receptive. Plant material can thus be available at any time for testing. Laboratory cultures of *Champia* provide test plants of similar preconditioning. Unialgal stock cultures of both males and females are easily maintained in separate, aerated, 1000-mL Erlenmeyer borosilicate flasks containing 800 to 900 mL of the culture medium. The culture medium is composed of natural seawater (or a 50-50 mixture of natural and artificial seawaters), to which additional nutrients are added.

X1.3 Several cultures of both males and females should be maintained simultaneously to keep a constant supply of plant material available. To maintain vigorous growth, initial stock cultures should be started periodically with approximately 0.5 to 1.0-cm tips of branches. Cultures are aerated gently through sterile, cotton-plugged, disposable, polystyrene 1-mL pipettes. Cultures are capped with foam plugs and aluminum foil and should be illuminated with 40 to 75 $\mu E\ m^{-2}\ s^{-1}$(approximately 200 to 500 fc) of cool-white fluorescent light on a 16:8, light:dark cycle. A dark period is essential for healthy reproductive structures. The use of irradiances greater than 50 $\mu E\ m^{-2}\ s^{-1}$ can result in varying degrees of abnormal morphology, depending on the type of culture incubator used (that is, the degree of reflectance). The preferred temperature is 22 to 24°C, and the preferred salinity is 28 to 32 g/kg. Media are changed once per week.

X1.4 Approximately half of the plants should be discarded (or placed in another culture vessel) with each weekly medium change in order to reduce the amount of biomass as the plants grow. At the end of approximately three weeks, there should be enough plant material to start testing. With this procedure, actively growing plants will be available continuously. The total number of cultures maintained will depend on the expected frequency of testing. The backup cultures should be kept in a separate location from the main cultures, and under the same culture conditions, although lower irradiances can be used.

X1.5 A stock culture should not be used as a source of test material if the plants appear to be stressed or undernourished. Under stress conditions, the tips of the branches will turn pink, and the older tissue will generally be much paler. Under nutrient deficiency conditions (usually resulting from too much plant material in the culture flask or too long a period since the last medium change), the entire plant will turn pale yellow. If the stress is severe enough, the older tissue (main axes) or, occasionally, the branch tips will turn white (evidence of necrotic tissue). The plants are not suitable for testing if cystocarps are present on females in the stock cultures. This usually occurs as a result of contamination by male tissue or water from male cultures; female cultures should thus be handled before male cultures.

X1.6 Stock cultures should be checked for their readiness for use in toxicity tests. First check for color uniformity of the tissue, for the presence of healthy sterile hairs and septa, and on the condition of the chloroplasts (Fig. X1.2). Females should be checked by examining the tips of a few branches under a compound microscope (100 times magnification or greater). Several trichogynes (reproductive hairs to which the spermatia attach) should be seen easily near the apex (Fig. X1.3). Although both sterile hairs and trichogynes occur on the apex, sterile hairs occur over the entire plant thallus. Sterile hairs are wider and generally much longer than trichogynes and appear hollow, except at their tip (Fig. X1.4). Male plants should be producing spermatia visibly. This is checked easily under a compound microscope. A mature sorus can be identified by looking at the edge of the thallus (Fig. X1.5) usually 1 to 2-cm back from the tip in healthy plants. Sori generally are thicker and lighter in color than the rest of the plant body.

X1.7 Once cultures are determined to be usable for toxicity testing, the tips of branches should be cut to their final size. For females, cut 7 to 10-mm branches, enough for five per treatment chamber (try to be consistent in the degree of branching). The cutting can be accomplished with fine-point forceps or a scalpel, with the plants in some dilution water in a petri dish. Repeat for males, except cut the branch segments to be 3-cm long and only one per treatment chamber. The males should be producing spermatia visibly (that is, two or more spermatial sori present on each branch segment cut, even if the segments must be larger than 3 cm). Prepare the female cuttings first in order to minimize the chance of contaminating them with water containing spermatia from the male cultures.

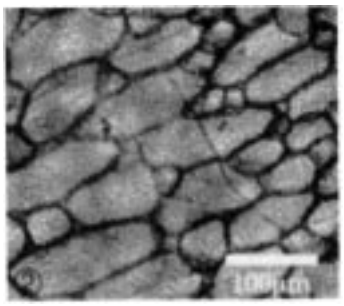

FIG. X1.2 Plant Surface Showing Healthy Chloroplasts

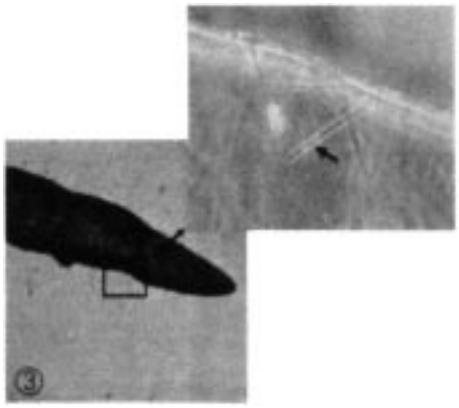

FIG. X1.3 Tip of Female Branch, Showing Hairs and Trichogyne (see Enlarged Area)

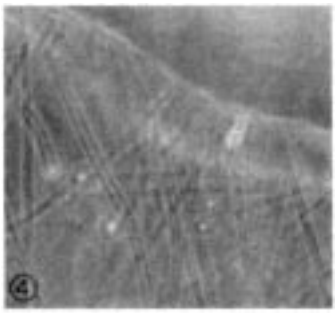

FIG. X1.4 Edge of Female Plant Surface, Showing Sterile Hairs

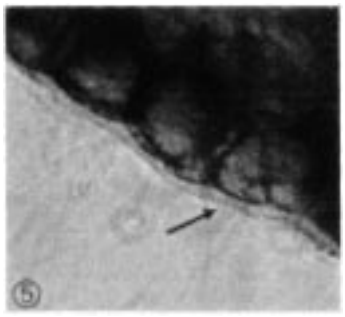

FIG. X1.5 Edge of Male Plant; Note Transition to Spermatial Sorus

X1.8 *Temperature, Salinity, Light, and Aeration*—The test exposure duration is two days, followed by a 5 to 7-day development period of the maturation of cystocarps (evidence of sexual reproduction). The exposure temperature should be between 22 and 24°C, and the test salinity should be 28 to 32 g/kg. For testing receiving waters or high concentrations of effluents, salinity will often be below the desired range and should be adjusted with artificial sea salts or hypersaline brine. The photoperiod should be a 16h:8h, light:dark cycle of 40 to 75 μE m^{-2} s^{-1} of cool-white fluorescent light. The conditions listed above are close to optimal and will result in sufficient cystocarp development during the to 5 to 7-day development period. Other conditions may yield acceptable results, but the recovery period may have to be changed to allow adequate cystocarp development.

X1.9 *Nutrients*—The nutrients added to the exposure medium are listed in Table 1. The recipe is modified for the exposure during toxicity testing by reduction or elimination of EDTA, omission of trace elements, and a general reduction in the concentration of the rest of the nutrients.

X1.10 Test chambers are not aerated during the exposure period. Chambers are either shaken at 100 r/min on a rotary shaker or hand-swirled briefly twice per day (even when shaken, a daily swirling helps to ensure spermatia suspension). Since spermatia are not motile, some water motion is essential. Aeration will enhance the growth rate of plants in the recovery bottles. Shaking at 100 r/min can be substituted, although cystocarp maturation may be delayed for a day or two.

X1.11 *Beginning the Test:*

X1.11.1 Plants can be prepared (cut) up to 1 h before they are needed. Set up and label the control and treatment chambers (four replicates are recommended), and fill them with the appropriate test solutions. The test begins when the test organism is first placed in the test solutions.

X1.11.2 Female branches should be added randomly to each test chamber. This is accomplished easily by first adding two female tips to each chamber, followed by two more, and then the final one. Add one male branch tip to one replicate for each treatment and control and repeat until a male branch has been added to all replicates. Branches should be handled only at the cut end.

X1.11.3 Test chambers should be placed under cool-white light and with a thermometer in a flask of water among the chambers. Temperature should be recorded daily.

X1.11.4 Chambers should be swirled gently twice per day. Alternately, shake continuously at 100 r/min on a shaker (swirling by hand once per day is still recommended).

X1.11.5 Media can be changed after 24 h, if the experimental design requires it. Label the vessels to be used for the development period (one for each test chamber) and add the appropriate medium.

X1.11.6 Remove the females after 48 h, and start the development period. Pour the contents of each test chamber into a clean, wide-mouth dish. Always start with the controls, and work from the lowest concentration to the highest. Take note of any necrotic tissue within any of the replicates. Necrosis will generally first appear on the male spermatial sori or on the cut ends. With forceps, remove females gently, briefly dip them in clean seawater to remove treatment water residue, and place them in recovery vessels. Discard the males. Place the vessels on a shaker or add aeration tubes (disposable, 1-mL pipettes work acceptably) and foam stoppers. If a shaker is used, reduce the volume of seawater to approximately one-half of the vessel volume to enhance the motion of the water.

X1.12 *Biological Data*—Count the number of cystocarps per female at the end of the development period (5 to 7 days), and record the data. As at the beginning of the development period, take note of any necrotic tissue, but also be aware of any morphological changes or differences in growth that might have occurred. This information is qualitative, but it may aid in data interpretation. Cystocarps are counted conveniently by placing the females between the inverted halves of a polystyrene petri dish with a small amount of seawater. This holds the

entire plant in approximately one focal plane. Using a stereomicroscope, the emergent cystocarps can be counted easily. Cystocarps are distinguished from young branches because they possess an apical ostiole (opening for spore release) and darkly pigmented spores (Fig. X1.6). One of the advantages to this test procedure is that if uncertainty exists concerning the identification of an immature cystocarp (Fig. X1.7), the plants can simply be aerated for slightly longer in the recovery vessels. No new cystocarps will form since the males have been removed; plants will only grow. Cystocarps will occasionally abort, and these should not be included in the systocarp counts. Aborted cystocarps (Fig. X1.8) are identified easily by their slightly darker pigmentation and angular shape. In addition, they will often begin to form a new branch at their apex after several additional days.

X1.13 *Other Data*—At the end of the exposure period, and again at the end of the recovery period, plants should be examined for the presence and extent of any necrotic tissue, morphological changes that might have occurred, and noticable differences in growth among the various treatments.

X1.14 *Acceptability of Test*—In addition to meeting the requirements listed in Section 12, test results should meet the following requirements:

X1.14.1 A test should not be accepted if control mortality (branches turn white) exists. Plants used in testing are from clonal material. It is rare that only some of the branches would die and not all.

X1.14.2 Plants from the controls and lowest exposure concentration should be in good physical condition. The branches should not be fragmented or necrotic. This could indicate either that the plants were unhealthy from the beginning of the test or that other problems, such as dirty glassware or poor quality brine (if used), exist.

X1.14.3 A test is not acceptable if the controls average fewer than 10 cystocarps per plant. Ideally, cystocarps should be clustered in groups (Fig. X1.9) on the controls.

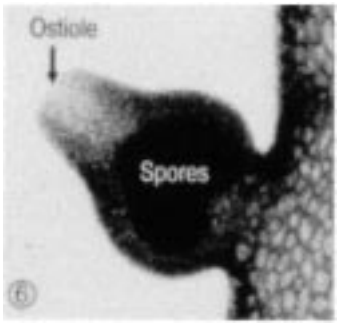

FIG. X1.6 Mature Cystocarp; Note Spores and Ostiole

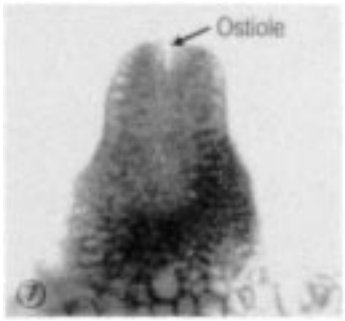

FIG. X1.7 Immature Cystocarp; Note Characteristic Shape and Ostiole

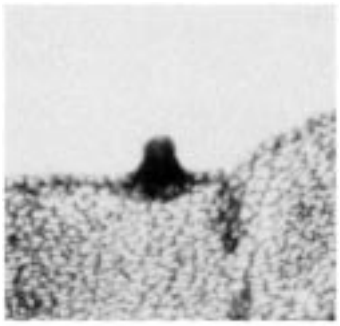

FIG. X1.8 Aborted Cystocarp

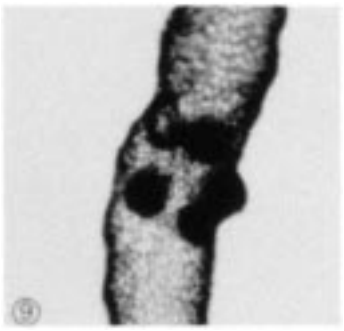

FIG. X1.9 Cluster of Immature Cystocarps

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