Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests

Supplement to Training Video

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NOTICE

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Foreword

This guide serves as a supplement to the video "Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests" (EPA, 2009). The methods illustrated in the video and described in this guide support the methods published in the U.S. Environmental Protection Agency's (EPA's) *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a), referred to as the Saltwater Chronic Methods Manual. The video and this guide provide details on preparing for and conducting the test based on the expertise of personnel at the following EPA Office of Research and Development (ORD) laboratories:

National Health and Environmental Effects Research Laboratory (NHEERL) – Atlantic Ecology Division in Narragansett, Rhode Island

NHEERL – Gulf Ecology Division in Gulf Breeze, Florida

National Exposure Research Lab (NERL) – Ecological Exposure Research Division (EERD) in Cincinnati, Ohio

This guide and its accompanying video are part of a series of training videos produced by EPA's Office of Wastewater Management. This Saltwater Series includes the following videos and guides:

"Mysid (*Americamysis bahia*) Survival, Growth, and Fecundity Toxicity Tests"

"Culturing *Americamysis bahia*"

"Sperm Cell Toxicity Tests Using the Sea Urchin, *Arbacia punctulata*"

"Red Algal (*Champia parvula*) Sexual Reproduction Toxicity Tests"

"Sheepshead Minnow (*Cyprinodon variegatus*) and Inland Silverside (*Menidia beryllina*) Larval Survival and Growth Toxicity Tests"

The Freshwater Series, released in 2006, includes the following videos and guides:

"*Ceriodaphnia* Survival and Reproduction Toxicity Tests"

"Culturing of Fathead Minnows (*Pimephales promelas*)"

"Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests"

All of these videos are available through the National Service Center for Environmental Publications (NSCEP) at 800 490-9198 or nscep@bps-lmit.com.

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Introduction

This guide accompanies the Environmental Protection Agency's (EPA's) video training for conducting red algal (*Champia parvula*) sexual reproduction toxicity tests (EPA, 2009). The test method is found in Section 16 of EPA's *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition* (EPA, 2002a). The test was developed by EPA's Office of Research and Development's (ORD's) National Health and Environmental Effects Research Laboratory-Atlantic Ecology Division (NHEERL-AED) in Narragansett, Rhode Island. The material presented in both the video and this guide summarizes the methods but does not replace a thorough review and understanding of the methods by laboratory personnel before conducting the test.

Background

Under the National Pollutant Discharge Elimination System (NPDES) program (Section 402 of the Clean Water Act), EPA uses toxicity tests to monitor and evaluate effluents for their toxicity to biota and their impact on receiving waters. By determining acceptable or safe concentrations for toxicants discharged into receiving waters, EPA can establish NPDES permit limitations for toxicity. These Whole effluent toxicity (WET) permit limitations regulate pollutant discharges on a whole effluent effect basis rather than by a chemical-specific approach only.

Whole effluent toxicity methods measure the synergistic, antagonistic, and additive effects of all the chemical, physical, and additive components of an effluent that adversely affect the physiological and biochemical functions of the test organisms. Therefore, healthy organisms and correct laboratory procedures are essential for valid test results. Laboratory personnel should be very familiar with the test methods and with red algae handling techniques before conducting a test.

This supplemental guide covers the procedures for conducting the test according to EPA's promulgated methods (*40 CFR Part 136;* EPA, 2002c) and also provides some helpful information that is not presented in the Saltwater Chronic Methods Manual (EPA, 2002a).

This guide summarizes methods developed at NHEERL-AED for estimating the chronic toxicity of marine or estuarine effluents and receiving waters on the sexual reproduction of the marine macroalga, *Champia parvula*. Males and females are exposed to effluents or receiving waters for 2 days, followed by a 5- to 7-day recovery period for the female plants in a control medium. Cystocarp production by the female, which indicates sexual reproduction, is used as the endpoint. The test results determine the effluent concentration causing a statistically significant reduction in the number of cystocarps formed.

This guide and accompanying video describe how the test is set up, initiated, terminated, and reviewed, including suggestions on maintaining healthy cultures of test organisms.

Culturing *Champia parvula*

There are three macroscopic stages in the life history of *Champia*. The adult plant body (thallus) is hollow, septate, and highly branched. Only the mature male and female plants are used in toxicity testing. Mature plants are illustrated in Figure 1.

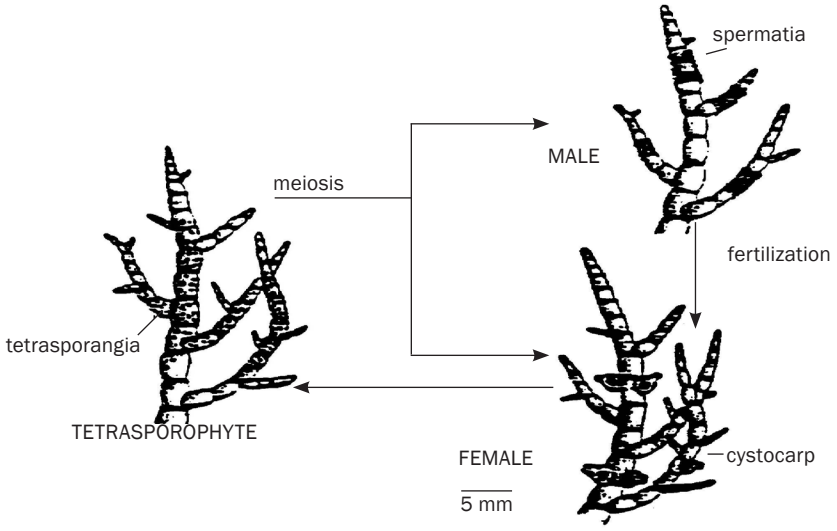
To keep a constant supply of plant material available, maintain several unialgal stock cultures of males and females simultaneously. Also, new cultures should be started weekly from excised branches so that cultures are available in different stages of development.

Culture Water

Natural seawater, or a 50-50 mixture of natural and artificial seawater, makes optimal culturing media. Seawater for cultures is filtered at least to 0.45 µm to remove most particulates and autoclaved for 30 minutes at 15 psi (120°C). Carbon stripping the seawater may be necessary before autoclaving to enhance

its water quality (EPA, 1990). Instructions for carbon stripping are provided in the Saltwater Chronic Methods Manual (EPA, 2002a). Nutrients should be added to the water to ensure healthy cultures. Recipes for the culturing medium and nutrient solutions are provided in Appendix A. The water temperature should be maintained at 23°C ± 1°C and the salinity at 30‰ ± 2‰

Figure 1. Life History of the Red Macroalga, *Champia parvula*. Left: Size and Degree of Branching in Female Branch Tips Used For Toxicity Tests



Source: EPA 1987.

Gently aerate the cultures. Change alternate cultures' medium every week so that if a stock solution should become contaminated, the entire batch will not be lost. While replenishing the medium, divide the growing algae in half with sharp forceps or discard half of the biomass to prevent overcrowding. New cultures also can be started at this time using 1 cm branch tips. Add nutrients using a pipet; NHEERL-AED has found a squeeze bottle is quick and easy to use. At the end of approximately three weeks, there should be enough plant material to conduct the test.

Photoperiod

The culture conditions should include a photoperiod 16 hours of light and 8 hours of darkness. The light level should not exceed 500 ft-candles (75 µE/m^2/s) and may have to be adjusted to that level, depending on the reflecting characteristics of the incubators.

Culture Vessels

Maintain stock cultures of males and females in separate, aerated, 1 L Erlenmeyer flasks containing 800 mL of the culture medium. All glass must be acid-stripped in 15 percent HCl and rinsed in deionized water before use because some detergent residues can be toxic to the *Champia*. At least every 6 months, the glass should be cleaned to remove organic materials that can build up on the surface. Always use sterile techniques when culturing the algae (i.e., autoclave all stock solutions and flame all tools before cutting or transferring plants) to guard against microalgal contamination.

Preparing Algae for Testing

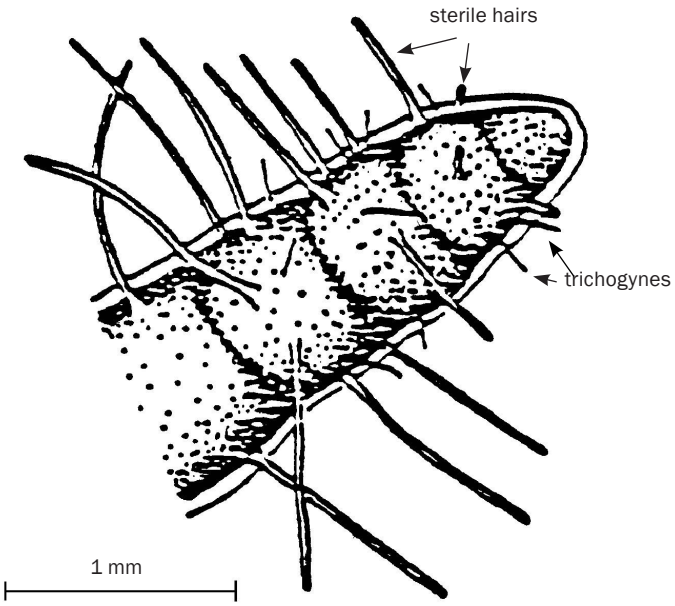
Examine the stock cultures to determine their readiness for testing. Place a few female branch tips in seawater in a petri dish, and examine them under a compound microscope to determine if trichogynes are present. An inverted scope works best with the petri dishes, although standard slides and microscopes also can be used. Trichogynes are the short, fine reproductive hairs to which the spermatia attach (see Figure 2). They should be seen easily near the apex of the branch tip. 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, where they seem to be plugged.

Males should be visibly producing spermatia. Sometimes, the presence of spermatia sori can be determined by placing some male tissue in a petri dish and holding it against a dark background. Mature sori can be easily identified under a microscope along the edge of the thallus. The sorus areas are generally thicker and lighter in color than the rest of the plant body. At higher magnification, the spermatia themselves can be seen (see Figures 3 and 4).

The readiness of the male stock culture can also be assessed by placing a portion of a female plant into a portion of the solution from the male culture for a few seconds. Under a microscope, numerous spermatia should be seen attached to the sterile hairs and trichogynes of the female plant (see Figure 5).

Once readiness is established for both males and females, the test can begin.

Figure 2. Apex of Branch of Female Plant, Showing Sterile Hairs and Reproductive Hairs (Trichogynes)



Sterile hairs are wider and generally much longer than trichogynes, and appear hollow except at the tip. Both types of hairs occur on the entire circumference of the thallus, but are seen easiest at the "edges." Receptive trichogynes occur only near the branch tips.

Source: EPA 1987.

Conducting the Test

Collecting the Algae

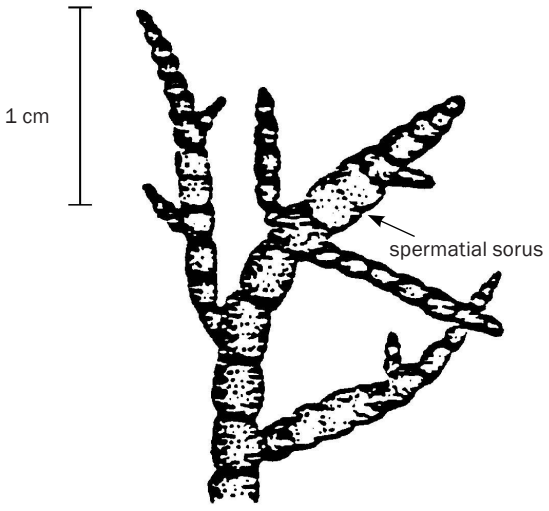
Prepare cuttings from the most healthy-looking plants. Prepare the female cuttings first to minimize the chances of contaminating them with water containing spermatia from the male stock cultures. Place each plant in a petri dish containing a small amount of seawater. Using a fine-point forceps or scalpel, prepare five cuttings from the female plants for each treatment replicate, severing the plant 7 – 10 mm from the ends of the branch. Try to be consistent in the degree of branching in the cuttings, since cystocarps form at the branch tips.

For male plants, use one cutting for each treatment replicate, severing the plant about 2 – 3 cm from the end of the branch. If there are few branches, or the spermatial sori appear sparse, larger male cuttings may be needed. The cuttings can be kept at room temperature for up to an hour.

Effluent Preparation

Effluent sampling should be conducted according to Section 8 of the Saltwater Chronic Methods Manual (EPA, 2002a) and any specific requirements of a NPDES permit. The effluent or receiving waters should be held at 0°C – 6°C until used for testing. Under the NPDES program, lapsed time from sample collection to first use in the test must not exceed 36 hours. Under special conditions or variances, samples may be held longer but should never be used for testing if held for more than 72 hours.

Figure 3. A Portion of the Male Thallus Showing Spermatial Sori. The Sorus Areas Are Generally Slightly Thicker and Somewhat Lighter in Color



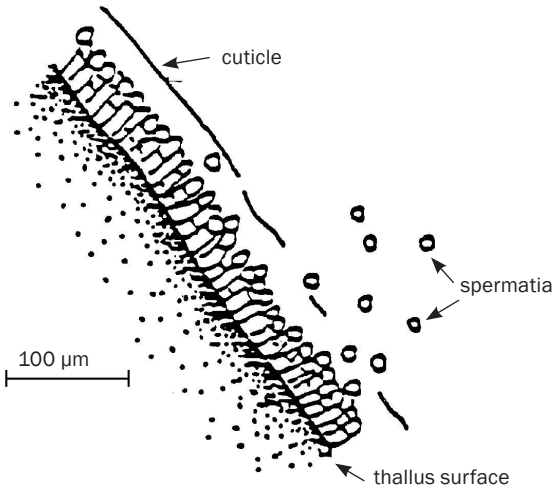
Source: EPA 1987.

Dilution Water

The type of dilution water used to make the test concentrations is dependent on the objectives of the test. Any specific requirements included in NPDES permits should be followed. The Saltwater Chronic Methods Manual (Section 7) provides the following guidelines:

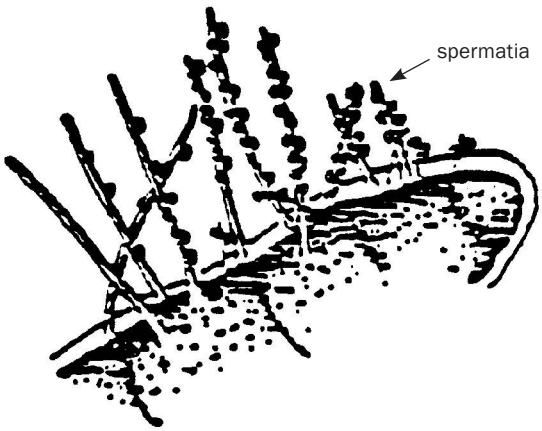
- *If the test is conducted to estimate the **absolute chronic toxicity of the effluent**, synthetic dilution water should be used. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.*
- *If the test is conducted to estimate the **chronic toxicity of the effluent in uncontaminated receiving waters,** the test can be conducted using a grab sample of the receiving waters collected outside the influence of the outfall, other uncontaminated waters, or standard dilution water with the same salinity as the receiving waters. If the cultures were maintained in different water than used for dilution water, a second set of control replicates should be conducted using the culture water.*
- *If the test is conducted to estimate the **additive or mitigating effects of the effluent on already contaminated receiving waters**, the test must be conducted using receiving waters collected outside the influence of the outfall. Controls should be conducted using both receiving water and culture water.*

Figure 4. A Magnified Portion of a Spermatial Sorus. Note the Rows of Cells that Protrude from the Thallus Surface



Source: EPA 1987.

Figure 5. Apex of a Branch on a Mature Female Plant That Was Exposed To Spermatia from a Male Plant



The sterile hairs and trichogynes are covered with spermatia. Note that few or no spermatia are attached to the older hairs (those more than 1 mm from the apex).

Source: EPA 1987.

Maintain the salinity of the test samples to 30‰ ± 2‰. To do this, effluent samples may need to be adjusted using hypersaline brine (HSB). A recipe for HSB is provided in Appendix A of this manual.

Approximately 1 hour before the test is to begin, adjust approximately 1 L of effluent to the test temperature of 23°C ± 1°C and maintain that temperature while preparing the test concentrations. To test a series of decreasing concentrations of effluent, use a dilution factor of ≥ 0.5. When starting with effluent that has 0‰ salinity, the maximum effluent concentration that can be prepared at 30‰ is 70 percent effluent. A table for preparing the samples is provided in Appendix A.

The Exposure Period

A 125 mL Erlenmeyer flask is used for each test chamber, but any clean container can be used. The test chambers should be labeled using colored tape and marking pens to identify each treatment and replicate. These should be placed in randomized positions for the duration of the test.

Under a hood, prepare five dilutions using a ≥ 0.5 dilution factor in 300 or 400 mL replicates. Approximately 1800 mL of effluent is required for a test conducted using a 0.5 dilution factor. This allows for enough of each prepared effluent concentration to provide four replicates at 100 mL and 400 mL for chemical analyses and water quality data. Record the water quality data on a form such as the one provided in Figure 6.

Figure 6. Receiving Water Data Form for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test.

Site: _____

Collection Date: _____

Test Date: _____

Locations	*Initial Salinity*	*Final Salinity*	*Source of Salts for Salinity Adjustment*[1]

[1]*Natural seawater, GP2 brine, GP2 salts, etc. (include some indication of amount.)*

Source: EPA, 2002a.

The 2-day exposure period starts when the algae are added to the test chambers. Add five female branches and one male branch to each prepared chamber. Pick up the branch at the base or cut end to avoid injuring the tips. The effluent must be in the test chamber before the algae are added.

Cover the chambers with aluminum foil or a foam stopper, exposing the cultures to 16 hours of cool white light and 8 hours of darkness each day for the 2-day exposure, as well as the 5- to 7-day recovery periods. Maintain the temperature at 23°C ± 1°C, and the salinity between 28‰ and 32‰ with the variance between chambers on any day maintained at ≤ 2‰.

Check on the chambers twice a day, and gently hand-swirl the chambers, or shake continuously at 100 rpm on a rotary shaker. Spermatia are not motile, so some motion is critical during the exposure period for reproduction to occur. If desired, the media can be changed after 24 hours. Record the temperature daily from a thermometer placed in a flask of water among the chambers.

pH should be measured in the effluent sample before any new test solutions are made to determine changes in the effluent sample.

Routine chemical and physical observations should be made during the test. Dissolved oxygen (DO) is measured at the beginning and end of each 24-hour exposure period in one test chamber at each concentration and in the control. Temperature, pH, and salinity are measured at the end of each 24-hour exposure period, also in one test chamber at each concentration and in the control. Temperature also should be monitored continuously, observed and recorded daily for at least two locations in the environmental control system or the samples. The locations for determining temperature should be sufficient to indicate any temperature variations in the environmental chamber.

The Recovery Period

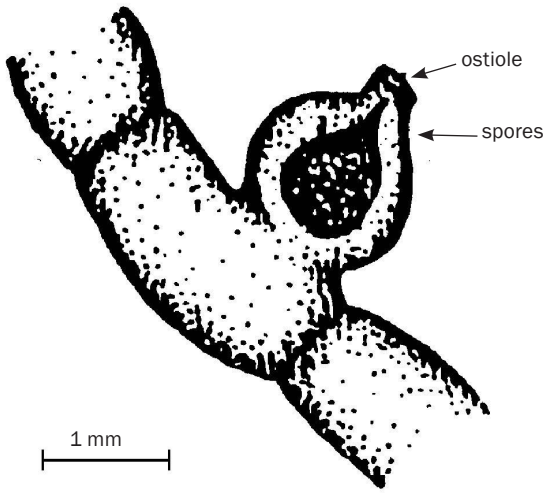
Prepare recovery bottles by labeling clean 100 – 200 mL vessels with the effluent concentrations tested, and fill them with 150 mL of natural seawater and nutrients. Smaller volumes can be used but may require changes of the medium to allow for adequate growth.

After the 48-hour exposure period, use forceps to gently remove all of the females from each test chamber, and place them into recovery bottles. When all the replicates have been transferred, place the vessels under cool white light and aerate or shake for the 5- to 7-day recovery period. Aeration will enhance the growth rate of plants in the recovery bottles, although adequate growth will occur using a shaker. Aerate using plastic tubes held in place by foam stoppers.

Terminating the Test

At the end of the recovery period, drain the chambers and remove the females with forceps, starting with the control plants and ending with those in the highest concentration. Place the female plants between the inverted halves of a petri dish containing a small amount of seawater, and count the cystocarps under a stereomicroscope. Cystocarps are distinguished from young branches by the darkly pigmented spores enclosed in the nodule, and the apical opening for spore release (ostiole). Figures 7 through 9 provide illustrations to help identify cystocarps.

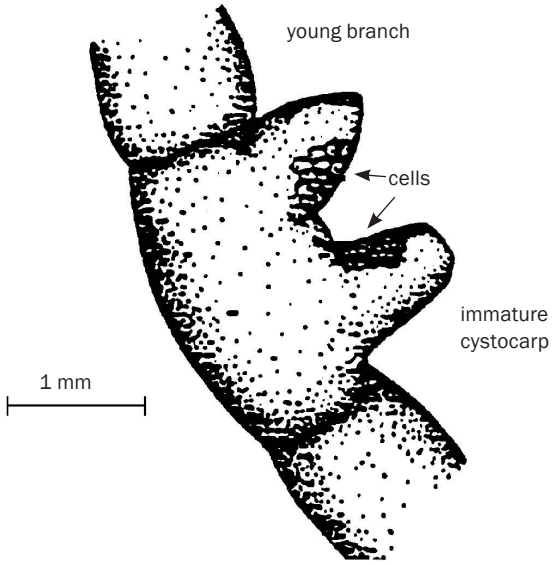
Figure 7. A Mature Cystocarp



In the controls and lower effluent concentrations, cystocarps often occur in clusters of 10 or 12.

Source: EPA 1987.

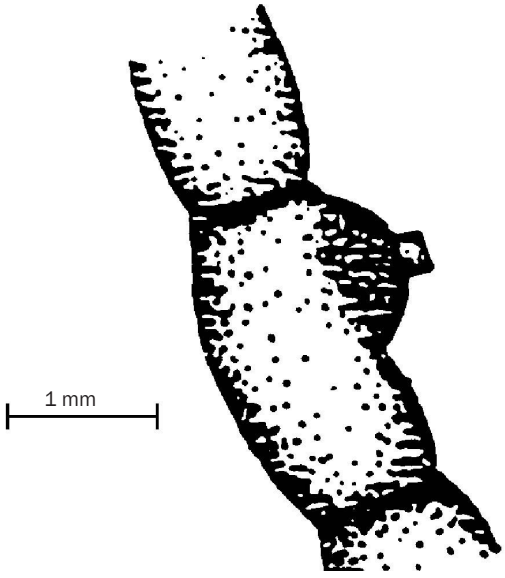
Figure 8. Comparison of a Very Young Branch and an Immature Cystocarp



Both the young branch and immature cystocarp can have sterile hairs. Trichogynes might or might not be present on a young branch, but are never present on an immature cystocarp. Young branches are more pointed at the apex and are made up of larger cells than immature cystocarps, and never have ostioles.

Source: EPA 1987.

Figure 9. An Aborted Cystocarp.



A new branch will eventually develop at the apex.

Source: EPA 1987.

If there is doubt about the identification of an immature cystocarp, aerate the plants a little longer in the recovery bottles. Within 24 to 48 hours, the suspected cystocarp will look more like a mature cystocarp or a young branch, or will have changed very little, if at all, indicating an aborted cystocarp. Occasionally cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and/or by the formation of a new branch at the apex. Dead plants lose their pigmentation and appear white.

Record all counts for the test on a form such as the one provided in Figure 10.

Figure 10. Cystocarp Data Sheet for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test

Collection Date: _____ Recovery Began (date): _____

Exposure Began (date): _____ Counted (date): _____

Effluent or Toxicant: _____

Treatment (% Effluent, mg/L, or receiving water sites)

Replicates	***Control***						
A 1							
2							
3							
4							
Mean							
B 1							
2							
3							
4							
Mean							
C 1							
2							
3							
4							
Mean							
D 1							
2							
3							
4							
Mean							
Overall Mean							

Temperature: _____

Salinity: _____

Light: _____

Source of Dilution Water: _____

Source: EPA, 1987.

Test Acceptability and Data Review

Test data are reviewed to verify that EPA's WET test methods' test acceptability criteria (TAC) requirements for a valid test have been met. The algal sexual reproduction test requires that several criteria be met before the test results are considered acceptable.

- Control plants should average 10 or more cystocarps per plant and survival in the control must be 80 percent or greater.
- Control and lowest-concentration exposed algae should be in good physical condition—for example, the branches should not be fragmented. Broken or fragmented branches could indicate that the plants were unhealthy or stressed from the beginning of the test.
- The results from the replicate control chambers should be similar.
- All replicates from the affected concentration chambers should show effect.

The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. In conjunction with this requirement, EPA has provided recommended guidance for concentration-response relationship review (EPA, 2000b).

EPA's promulgated toxicity testing method manuals (EPA, 2002a, b) recommend the use of point estimation technique approaches for calculating endpoints for effluent toxicity tests under the NPDES program. The promulgated methods also require a data review of toxicity data and concentration-response data, and require calculating the percent minimum significant difference (PMSD) when point estimation (e.g., LC_{50}, IC_{25}) analyses are not used. EPA specifies the PMSD must be calculated when NPDES permits require sub-lethal hypothesis testing. EPA also requires that variability criteria be applied as a test review step when NPDES permits require sub-lethal hypothesis testing endpoints (i.e., no observed effect concentration [NOEC] or lowest observed effect concentration [LOEC]) and the effluent has been determined to have no toxicity at the permitted receiving water concentration (EPA, 2002b). This reduces the within-test variability and increases statistical sensitivity when test endpoints are expressed using hypothesis testing rather than the preferred point estimation techniques.

Citations and Recommended References

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EPA references are available online at **www.epa.gov/npdes**.

If you need additional copies of this document, you can download it at: **www.epa.gov/npdes/wqbasedpermitting**.

Glossary

Acute toxicity. An adverse effect measured on a group of test organisms during a short-term exposure in a short period of time (96 hours or less in toxicity tests). The effect can be measured in lethality or any variety of effects.

Champia parvula. The scientific name for red algae. *Champia parvula* have soft, gelatinous, pinkish red, much-branched fronds that are densely matted, with blunt apices, to 100 mm high. Their axes are segmented, with nodal diaphragms. The segments are about as broad as long, filled with a watery mucilage. Red algae are found epiphytic on smaller algae in lower intertidal pools. They are found widely distributed in the Atlantic and Pacific marine environments.

Chronic toxicity. An adverse effect that occurs over a long exposure period. The effect can be lethality, impaired growth, reduced reproduction, etc.

Diluent water. Dilution water used to prepare the effluent concentrations.

Effluent concentrations. Concentrations or dilutions of an effluent sample to which test organisms are exposed to determine the biological effects of the sample on the test organism.

Effluent sample. A representative collection of the discharge that is to be tested.

Hypothesis testing. Technique (e.g., Dunnett's test) that determines what concentration is statistically different from the control. Endpoints determined from hypothesis testing are NOEC and LOEC.

IC_{25} (Inhibition Concentration, 25%). The point estimate of the toxicant concentration that would cause a 25% reduction in a non-quantal biological measurement (e.g., reproduction or growth) calculated from a continuous model.

LC_{50} (Lethal Concentration, 50%). The concentration of toxicant or effluent that would cause death to 50% of the test organisms at a specific time of observations (e.g., 96-hour LC_{50}).

Lowest Observed Effect Concentration (LOEC). The LOEC is the lowest concentration of toxicant to which organisms are exposed in a test, which causes statistically significant adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically significantly different from the control). The definitions of NOEC and LOEC assume a strict dose-response relationship between toxicant concentration and organism response.

Minimum Significant Difference (MSD). The MSD is the magnitude of difference from the control where the null hypothesis is rejected in a statistical test comparing a treatment with a control. MSD is based on the number of replicates, control performance and power of the test. MSD is often measured as a percent and referred to as PMSD.

No Observed Effect Concentration (NOEC). The NOEC is the highest tested concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effect on the test organism (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically significantly different from the controls). NOECs calculated by hypothesis testing are dependent upon the concentrations selected.

NPDES (National Pollutant Discharge Elimination System) Program. The national program for issuing, modifying, revoking and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under Sections 307, 318, 402, and 405 of the Clean Water Act.

Point Estimation Techniques. This technique is used to determine the effluent concentration at which adverse effects (e.g., fertilization, growth or survival) occurred, such as Probit, Interpolation Method, Spearman-Karber. For example, a concentration at which a 25% reduction in reproduction and survival occurred.

Receiving Water Concentration (RWC). The RWC is the concentration of a toxicant or the parameter toxicity in the receiving water (i.e., riverine, lake, reservoir, estuary or ocean) after mixing.

Toxicity test. A test to measure the toxicity of a chemical or effluent using living organisms. The test measures the degree of response of an exposed organism to a specific chemical or effluent.

WET (Whole effluent toxicity). The total toxic effect of an effluent measured directly with a toxicity test.

Appendix A: Nutrients and Media

The following instructions for nutrients are provided in the Saltwater Chronic Methods Manual (EPA, 2002a). Table A-1 lists the additional nutrients to be added to natural or artificial seawater for stock cultures and test media. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 minutes before the vitamins are added. Adjust the solution to about pH 2 before autoclaving to minimize the possibility of precipitation.

Table A-1. Nutrient Stock Solution

Nutrient Stock Solution[a]	*Amount/L Concentrated Nutrient Stock Solution*	
	Stock Solution for Culture Medium	*Stock Solution for Test Medium*
$NaNO_3$	6.35 g	1.58 g
$NaH_2PO_4 \cdot H_2O$	0.64 g	0.16 g
$Na_2EDTA \cdot 2\ H_2O$	133 mg	—
$Na_3C_6H_5O_7 \cdot 2\ H_2O$	51 mg	12.8 mg
Iron[b]	9.75 mL	2.4 mL
Vitamins[c]	10 mL	2.5 mL

[a] *Add 10 mL of appropriate nutrient stock solution per liter of culture or test medium.*

[b] *A stock solution of iron is made that contains 1 mg iron/mL. Ferrous or ferric chloride can be used.*

[c] *A vitamin stock solution is made by dissolving 4.88 g thiamine HCl, 2.5 mg biotin, and 2.5 mg B_{12} in 500 mL deionized water. Adjust vitamin stock to approximately pH 4, divide into 10 mL subsamples, and autoclave for 2 minutes before it is added to the nutrient stock solution.*

Preparing Hypersaline Brine (HSB)

Background

Champia parvula cannot be cultured in 100% artificial seawater. However, 100% artificial seawater can be used during the 2-day exposure period. This allows 100% effluent to be tested.

Salinity adjustments are a vital part of using marine and estuarine species for toxicity testing. The majority of industrial and sewage treatment effluents entering marine and estuarine waters contain little or no measurable salts. Therefore, the salinity of these effluents must be adjusted before exposing estuarine or marine plants and animals to the solutions. The salinity of the effluent can be adjusted by adding HSB prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in table below), or dry GP2 salts (also below). Adjust the salinity of the effluent to 30‰. Control solutions should be prepared with the same percentage of natural seawater and at the same salinity as the effluent solutions.

Constant salinity should be maintained across all treatments throughout the test for quality control. Matching the test solutions' salinity to the expected receiving water's salinity may require salinity adjustments. EPA NHEERL-AED uses HSB, prepared from filtered natural seawater, to adjust exposure solution salinities.

HSB has several advantages over artificial sea salts that make it more suitable for use in toxicity testing. Concentrated brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of test organisms. It may be held for prolonged periods without any apparent degradation. Brine may be added directly to the effluent to increase the salinity, or may be used as control water by diluting to the

desired salinity with deionized water. The brine can be made from any high quality, filtered seawater supply through simple heating and aerating.

Table A-2. GP2 Artificial Seawater for Use in Conjunction with Natural Seawater for the Red Macroalga, *Champia parvula*, Sexual Reproduction Toxicity Test

Compound	*Concentration (g/L)*	*Amount (g) Required for 20 L*
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$[a]	0.17	3.40

The original formulation calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), all of the other salts can be autoclaved together. Since no nutrients are added until needed, autoclaving is not critical for effluent testing. To minimize microalgal contamination, the artificial seawater should be autoclaved when used for stock cultures. Autoclaving (120°C) should be for at least 10 minutes for 1-L volumes, and 20 minutes for 10- to 20-L volumes.

Artificial seawater should be prepared in 10- to 20-L batches. Effluent salinity adjustment to 30‰ can be made by adding the appropriate amount of dry salts from this formulation, by using a triple-strength brine prepared from this formulation, or by using a 100‰ salinity brine prepared from natural seawater.

Nutrients listed in Table A-1 should be added to the artificial seawater in the same concentration described for natural seawater.

[a] *A stock solution of 68 mg/mL sodium bicarbonate is prepared by autoclaving it as a dry powder, and then dissolving it in sterile deionized water. For each liter of GP2, use 2.5 mL of this stock solution.*

Source: EPA, 2002a. Modified from Spotte et al., 1984. Constituents salts and concentrations were taken from EPA 1990.

Generating the Brine

The ideal container for making brine from natural seawater has a high surface-to-volume ratio, is made of a non-corrosive material, and is easily cleaned. Shallow fiberglass tanks are ideal.

Collect high-quality (and preferably high-salinity) seawater on an incoming tide to minimize the possibility of contamination. Special care should be used to prevent any toxic materials from coming in contact with the seawater. The water should be filtered to at least 10 µm before placing into the brine tank. Thoroughly clean the tank, aeration supply tube, heater, and any other materials that will be in direct contact with the brine before adding seawater to the tank. Use a good-quality biodegradable detergent, followed by several thorough deionized-water rinses. Fill the tank with seawater, and slowly increase the temperature to 40°C. If a heater is immersed directly into the seawater, make sure that the heater components will not corrode or leach any substances that would contaminate the brine. A thermostatically controlled heat exchanger made from fiberglass works well.

Aeration prevents temperature stratification and increases the rate of evaporation. Use an oil-free air compressor to prevent contamination. Evaporate the water for several days, checking daily (or more or less often, depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and the temperature does not exceed 40°C. If these changes are exceeded, irreversible changes in the brine's properties may occur. One such change noted in original studies at ERL-N was a reduction in the alkalinity

of seawater made from brine with salinity greater than 100‰, and a resulting reduction in the animals' general health. Additional seawater may be added to the brine to produce the volume of brine desired.

When the desired volume and salinity of brine is prepared, filter the brine through a 10-µm filter and pump or pour it directly into portable containers (5-gallon cubitainers or polycarbonate water cooler jugs are most suitable). Cap the containers, and record the measured salinity and the date the brine was generated. Store the brine in the dark at room temperature until used.

Salinity Adjustments Using HSB

To calculate the volume of brine (V_b) to add to 0‰ sample to produce a solution at certain salinity (S_f), use this equation:

$$V_b * S_b = S_f * V_f$$

Where V_b = volume of brine, mL
S_b = salinity of brine, ‰
S_f = final salinity, ‰
V_f = final volume, mL (brine brought to this volume with 0 ‰ sample).

Table A-3 gives volumes needed to make 30‰ test solutions from effluent (0‰), deionized water, and 100‰ HSB. At 30‰ salinity, the highest achievable concentration is 70% effluent.

Table A-3. Preparation of Test Solutions at a Salinity of 30‰ Using HSB for a Final Test Concentration Volume of 1000 mL.

Exposure Concentration (%)	Effluent (0 ‰) (mL)	Deionized Water (mL)	Hypersaline Brine (100 ‰) (mL)
70	700	—	300
25	250	450	300
7	70	630	300
2.5	25	675	300
0.7	7	693	300
Control	—	1,000	—

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Appendix B: Apparatus and Equipment

Air lines, and air stones. For aerating cultures, brood chambers, and holding tanks, and supplying air to test solutions with low DO.

Air pump. For oil-free air supply.

Balance. Analytical, capable of accurately weighing to 0.00001 g.

Beakers, Class A. Borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

Bottles. Borosilicate glass or disposable polystyrene cups (200 – 400 mL) for use as recovery vessels.

Compound microscope. For examining the condition of plants.

Count register. 2-place for recording cystocarp counts.

Dissecting (stereomicroscope) microscope. For counting cystocarps.

Drying oven. To dry glassware.

Erlenmeyer flasks, 250 mL, or 200 mL disposable polystyrene cups, with covers. For use as exposure chambers.

Environmental chamber or equivalent facility with temperature control (23 ± 1°C).

Facilities for holding and acclimating test organisms.

Filtering apparatus. For use with membrane filters (47 mm).

Forceps, fine-point, stainless steel. For cutting and handling branch tips.

Laboratory red macroalga, *Champia parvula*, culture unit. To test effluent or receiving water toxicity, sufficient number of sexually mature male and female plants must be available.

Meters: pH and DO, and specific conductivity. For routine physical and chemical measurements.

Micropipettors, digital, 200 and 1000 µL. To make dilutions.

Pipet bulbs and filters. Propipet®, or equivalent.

Pipets, automatic. Adjustable 1 – 100 mL.

Pipets, serological. 1 – 10 mL, graduated.

Pipets, volumetric. Class A, 1 – 100 mL.

Reference weights, Class S. For checking performance of balance.

Refractometer or other method. For determining salinity.

Rotary shaker. For incubating exposure chambers (hand-swirling twice a day can be substituted).

Samplers. Automatic samplers, preferably with sample cooling capability, that can collect a 24-hour composite sample of 1 L.

Thermometers. National Bureau of Standards Certified (see EPA 2002a). Used to calibrate laboratory thermometers.

Thermometers. Bulb-thermograph or electronic-chart type for continuously recording temperature.

Thermometers, glass or electronic, laboratory grade. For measuring water temperatures.

Water purification system. Millipore® Milli-Q® deionized water or equivalent.

Wash bottles. For deionized water, for washing organisms from containers and for rinsing small glassware and instrument electrodes and probes.

Volumetric flasks and graduated cylinders. Class A, borosilicate glass or non-toxic plastic labware, 10 – 1000 mL for making test solutions.

Appendix C: Reagents and Consumable Materials

Aluminum foil, foam stoppers, or other closures. To cover cultures and test flasks.

Artificial seawater. A slightly modified version of the GP2 medium (Spotte, et al., 1984) has been used successfully to perform the red macroalga sexual reproduction test. The preparation of artificial seawater (GP2) is described in Table A-2.

Buffers pH 4, pH 7, and pH 10. (Or as per instructions of instrument manufacturer) for standards and calibration check.

Data sheets (one set per test). For data recording (see Figures 6 and 10).

Disposable tips for micropipettors.

Effluent, receiving water, and dilution water. Test waters, including effluent, receiving, and dilution water should be analyzed to ensure its quality prior to using in tests. Dilution water containing organisms that might prey upon or otherwise interfere with the test organisms should be filtered through a fine mesh (with 150 µm or smaller openings).

Laboratory quality assurance samples and standards. For the above methods.

Markers, waterproof. For marking containers, etc.

Mature red macroalga, *Champia parvula*, plants.

Petri dishes, polystyrene. To hold plants for cystocarp counts and to cut branch tips. Other suitable containers may be used.

pH buffers pH 4, pH 7, and pH 10. (Or as per instructions of instrument manufacturer) for standards and calibration check.

Reagent water. Distilled or deionized water that does not contain substances which are toxic to the test organisms.

Reference toxicant solutions. Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCl), cadmium chloride ($CdCl_2$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

Saline test and dilution factor. The use of natural seawater is recommended for this test. A recipe for the nutrients that must be added to the natural seawater is given in Table A-1. The salinity of the test water must be 30‰ and vary no more than ± 2‰ among the replicates. If effluent and receiving water tests are conducted concurrently, the salinity of these tests should be similar.

The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Therefore, exposure of the red macroalga, *Champia parvula*, to effluents will usually require adjustments in the salinity of the test solutions. Although the red macroalga, *Champia parvula*, cannot be cultured in 100% artificial seawater, 100% artificial seawater can be used during the 2-day exposure period. This allows 100% effluent to be tested. It is important to maintain a constant salinity across all treatments. The salinity of the effluent can be adjusted by adding HSB prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in Table A-2), or dry GP2 salts (Table

A-2), to the effluent to provide a salinity of 30‰. Control solutions should be prepared with the same percentage of natural seawater and at the same salinity (using deionized water adjusted with dry salts, or brine) as used for the effluent dilutions.

Sample containers. For sample shipment and storage.

Tape, colored. For labeling test chambers.

Appendix D: Summary of Test Conditions and Test Acceptability Criteria for the Red Macroalga, *Champia parvula*, Sexual Reproduction Test With Effluents and Receiving Waters

(Note: this test is not listed at 40 CFR Part 136 for nationwide use)

Test type	Static, non-renewal *(required)*
Salinity	30‰ ± 2 ‰ of the selected test salinity *(recommended)*
Temperature (C°)	23°C ± 1°C *(recommended)*
Light source	Cool-white fluorescent lights *(recommended)*
Light intensity	About 75 µE/m^2/s (500 ft-c) *(recommended)*
Photoperiod	16 hr light, 8 hr dark *(recommended)*
Test chamber size	200 mL polystyrene cups (with covers) or 250 mL Erlenmeyer flasks *(recommended)*
Test solution volume	100 mL *(minimum required)*
Number of organisms per test chamber	5 female branch tips and one male plant *(recommended)*
Number of replicates per concentration	4 *(3 required minimum)*
Number of organisms per concentration	24 *(18 required minimum)*
Aeration	None; chambers are either shaken at 100 rpm on a rotary shaker or hand-swirled twice a day
Dilution water	30‰ salinity natural seawater, or a combination of 50% of 30‰ salinity natural seawater and 50% of 30‰ salinity GP2 artificial seawater
Test concentrations	Effluents: 5 and a control *(required minimum)*
Receiving waters	100% receiving water (*or minimum of 5)* and a control *(recommended)*
Dilution factor	Effluents: ≥ 0.5 *(recommended)* Receiving Waters: None or ≥ 0.5 *(recommended)*
Test duration	2-day exposure to effluent followed by 7-day recovery period in control medium for cystocarp development *(required)*
Endpoints	Reduction in cystocarp production compared to controls *(required)*
Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls *(required)*
Sampling requirements	For on-site tests, one sample collected at test initiation, and used within 24 hr of the time it is removed from the sampling device. For off-site test, holding time must not exceed 36 hr before test use. *(required)*
Sample volume required	2 L per test *(recommended)*

Source: EPA, 2002a. Saltwater Chronic Methods Manual.

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