SECTION 16

TEST METHOD

RED MACROALGA, *CHAMPIA PARVULA,* SEXUAL REPRODUCTION TEST METHOD 1009.0

16.1 SCOPE AND APPLICATION

16.1.1 CAUTION: The Red Macroalga, *Champia parvula*, Reproduction Test Method 1009.0 is not listed at 40 CFR Part 136 for nationwide use.

16.1.2 This method, adapted in part from USEPA (1987f) measures the effects of toxic substances in effluents and receiving water on the sexual reproduction of the marine red macroalga, *Champia parvula*. The method consists of exposing male and female plants to test substances for two days, followed by a 5-7 day recovery period in control medium, during which the cystocarps mature.

16.1.3 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

16.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, highly volatile and highly degradable toxicants present in the source may not be detected in the test.

16.1.5 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

16.2 SUMMARY OF METHOD

16.2.1 Sexually mature male and female branches of the red macroalga, *Champia parvula*, are exposed in a static system for 2 days to different concentrations of effluent, or to receiving water, followed by a 5 to 7 day recovery period in control medium. The recovery period allows time for the development of cystocarps resulting from fertilization during the exposure period. The test results are reported as the concentration of the test substance which causes a statistically significant reduction in the number of cystocarps formed.

16.3 INTERFERENCES

16.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

16.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

16.3.3 Adverse effects of high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

16.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

16.4 SAFETY

16.4.1 See Section 3, Safety and Health.

16.5 APPARATUS AND EQUIPMENT

16.5.1 Facilities for holding and acclimating test organisms.

16.5.2 Laboratory red macroalga, *Champia parvula*, culture unit -- see culturing methods below. To test effluent or receiving water toxicity, sufficient numbers of sexually mature male and female plants must be available.

16.5.3 Samplers -- automatic samplers, preferably with sample cooling capability, that can collect a 24-h composite sample of 1 L.

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

16.5.5 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

16.5.6 Air pump -- for oil-free air supply.

16.5.7 Air lines, and air stones -- for aerating cultures.

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

16.5.9 Reference weights, Class S -- for checking performance of balance.

16.5.10 Meter, pH -- for routine physical and chemical measurements.

16.5.11 Dissecting (stereoscope) microscope -- for counting cystocarps.

16.5.12 Compound microscope -- for examining the condition of plants.

16.5.13 Count register, 2-place -- for recording cystocarp counts.

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

16.5.15 Drying oven -- to dry glassware.

16.5.16 Filtering apparatus -- for use with membrane filters (47 mm).

16.5.17 Refractometer -- for determining salinity.

16.5.1 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

16.5.19 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

16.5.20 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, l979b) -- to calibrate laboratory thermometers.

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

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

16.5.23 Bottles -- borosilicate glass or disposable polystyrene cups (200-400 mL) for use as recovery vessels.

16.5.24 Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.

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

16.5.26 Micropipettors, digital, 200 and 1000 µL – to make dilutions.

16.5.27 Pipets, volumetric -- Class A, 1-100 mL.

16.5.28 Pipettor, automatic -- adjustable, 1-100 mL.

16.5.29 Pipets, serological -- 1-10 mL, graduated.

16.5.30 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

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

16.6 REAGENTS AND CONSUMABLE MATERIALS

16.6.1 Mature red macroalga, *Champia parvula*, plants -- see Subsection 16.6.14 below.

16.6.2 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

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

16.6.4 Disposable tips for micropipettors.

16.6.5 Aluminum foil, foam stoppers, or other closures -- to cover culture and test flasks.

16.6.6 Tape, colored -- for labeling test chambers.

16.6.7 Markers, waterproof -- for marking containers, etc.

16.6.8 Data sheets (one set per test) -- for data recording.

16.6.9 Buffers, pH 4, pH 7, and pH l0 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

16.6.10 Laboratory quality assurance samples and standards for the above methods.

16.6.11 Reference toxicant solutions see Section 4, Quality Assurance.

16.6.12 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

16.6.13 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

16.6.13.1 Saline test and dilution water -- 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 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.

16.6.13.2 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 two 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 hypersaline brine (HSB) prepared from natural seawater (100‰), concentrated (triple strength) salt solution (GP2 described in Table 2), or dry GP2 salts (Table 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.

16.6.13.3 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 2.

TABLE 1. NUTRIENTS TO BE ADDED TO NATURAL SEAWATER AND TO ARTIFICIAL SEAWATER (GP2) DESCRIBED IN TABLE 2. THE CONCENTRATED NUTRIENT STOCK SOLUTION IS AUTOCLAVED FOR 15 MINIMUM (VITAMINS ARE AUTOCLAVED SEPARATELY FOR 2 MINIMUM AND ADDED AFTER THE NUTRIENT STOCK SOLUTION IS AUTOCLAVED). THE pH OF THE SOLUTION IS ADJUSTED TO APPROXIMATELY pH 2 BEFORE AUTOCLAVING TO MINIMIZE THE POSSIBILITY OF PRECIPITATION

	Amount of Reagent Per Liter of Concentrated Nutrient Stock Solution	
	Stock Solution For Culture Medium	Stock Solution For Test Medium
Nutrient Stock Solution[1]		
$NaNO_3$	6.35 g	1.58 g
$NaH_2P0_4 \cdot H_2O$	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[2]	9.75 mL	2.4 mL
Vitamins[3]	10 mL	2.5 mL

1 Add 10 mL of appropriate nutrient stock solution per liter of culture or test medium.

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

3 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 approximately pH 4 before autoclaving 2 min. It is convenient to subdivide the vitamin stock into 10 mL volumes in test tubes prior to autoclaving.

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR USE IN CONJUNCTION WITH NATURAL SEAWATER FOR THE RED MACROALGA, *CHAMPIA PARVULA*, CULTURING AND TOXICITY TESTING[1,2,3,4,5,6,7]

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
$NaHCO_3$	0.17	3.40

1 Modified GP2 from Spotte et al. (1984).

2 The constituent salts and concentrations were taken from USEPA (l990b).

3 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 a least 10 minimum for 1-L volumes, and 20 minimum for 10-to-20-L volumes.

4 Prepare in 10-L to 20-L batches.

5 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.

6 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.

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

16.6.14 TEST ORGANISMS RED MACROALGA, *CHAMPIA PARVULA*

16.6.14.1 Cultures

16.6.14.1.1 Mature plants are illustrated in Figure 1. The adult plant body (thallus) is hollow, septate, and highly branched. New cultures can be propagated asexually from excised branches, making it possible to maintain clonal material indefinitely.

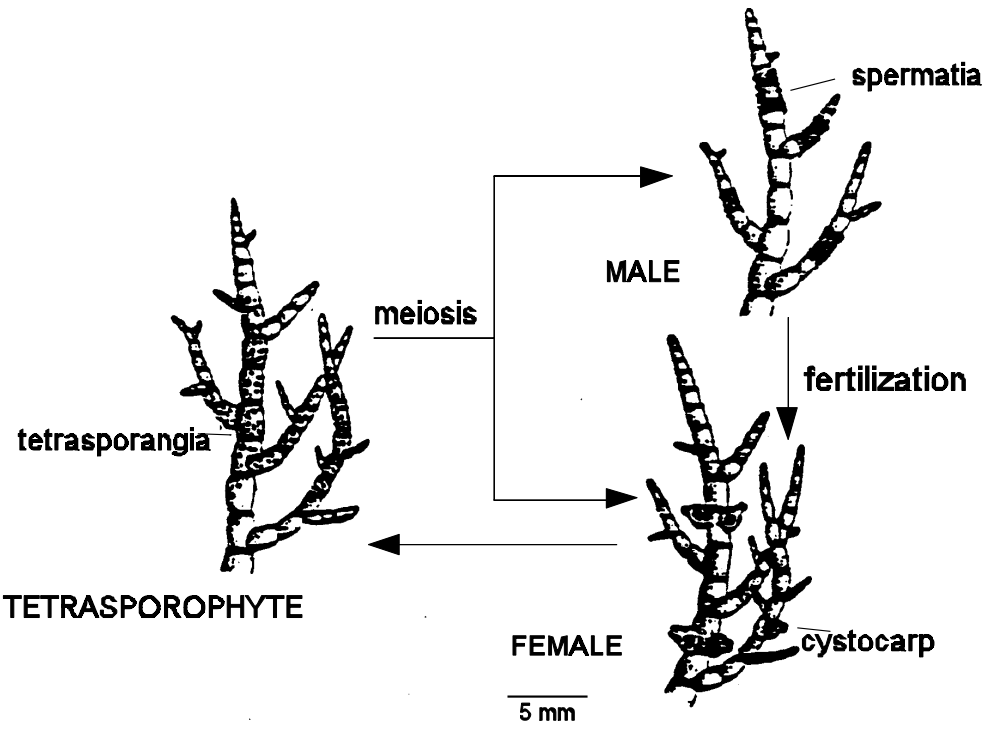

Figure 1. Life history of the red macroalga, *Champia parvula*. Upper left: Size and degree of branching in female branch tips used for toxicity tests. From USEPA (1987f).

16.6.14.1.2 Unialgal stock cultures of both males and females are maintained in separate, aerated 1000 mL Erlenmeyer flasks containing 800 mL of the culture medium. All culture glass must be acid-stripped in 15% HCl and rinsed in deionized water after washing. This is necessary since some detergents can leave a residue that is toxic to the red macroalga, *Champia parvula*. Periodically (at least every 6 months) culture glassware should be baked in a muffle furnace to remove organic material that may 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.

16.6.14.1.3 The culture medium is made from natural seawater to which additional nutrients are added. The nutrients added are listed in Table 1. Almost any nutrient recipe can be used for the red macroalga, *Champia parvula*, cultured in either natural seawater or a 50-50 mixture of natural and artificial seawaters. Healthy, actively growing plants are the goal, not a standard nutrient recipe for cultures.

16.6.14.1.4 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 about twenty 0.5 to l.0 cm branch tips. Cultures are gently aerated through sterile, cotton-plugged, disposable, polystyrene l mL pipettes. Cultures are capped with foam plugs and aluminum foil and illuminated with ca. 75 $\mu E/m^2/s$ (500 ft-c) of cool-white fluorescent light on a 16:8 h light:dark cycle. Depending on the type of culture chamber or room used, i.e.,

the degree of reflected light, the light levels may have to be adjusted downward. The temperature is 22 to 24°C and the salinity 28-30‰. Media are changed once a week.

16.6.14.1.5 Prior to use in toxicity tests, stock cultures should be examined to determine their condition. Females can be checked by examining a few branch tips under a compound microscope (100 X or greater). Several trichogynes (reproductive hairs to which the spermatia attach) should be easily seen near the apex (Figure 2).

16.6.14.1.6 Male plants should be visibly producing spermatia. This can be checked by placing some male tissue in a petri dish, holding it against a dark background and looking for the presence of spermatial sori. Mature sori can also be easily identified by looking along the edge of the thallus under a compound microscope (Figures 3 and 4).

16.6.14.1.7 A final, quick way to determine the relative "health" of the male stock culture is to place a portion of a female plant into some of the water from the male culture for a few seconds. Under a compound microscope numerous spermatia should be seen attached to both the sterile hairs and the trichogynes (Figure 5).

16.6.14. Culture medium prepared from natural seawater is preferred (Table 1). However, as much as 50% of the natural seawater may be replaced by the artificial seawater (GP2) described in Table 2.

16.6.14.2.1 Seawater for cultures is filtered at least to 0.45 µm to remove most particulates and then autoclaved for 30 minute at 15 psi (120°C). Carbon stripping the seawater may be necessary before autoclaving to enhance its water quality (USEPA, 1990b). This is done by adding 2 g activated carbon per liter of seawater and stirring on a stir plate for 2 h. After stirring filter through a Whatman number 2 filter, then through a 0.45 membrane filter. The culture flasks are capped with aluminum foil and autoclaved dry, for 10 minute. Culture medium is made up by dispensing seawater into sterile flasks and adding the appropriate nutrients from a sterile stock solution.

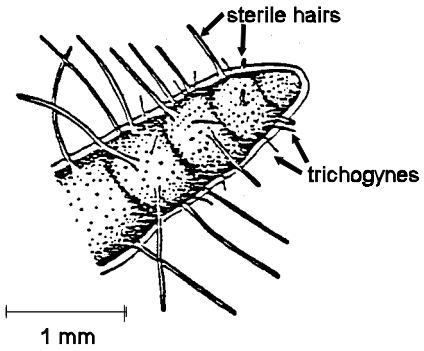

Figure 2. Apex of branch of female plant, showing sterile hairs and reproductive hairs (trichogynes). Sterile hairs are wider and generally much longer than trichogynes, and appear hollow except at the tip. Both types of hairs occur on the entire circumference of the thallus, but are seen easiest at the "edges." Receptive trichogynes occur only near the branch tips. From USEPA (1987f).

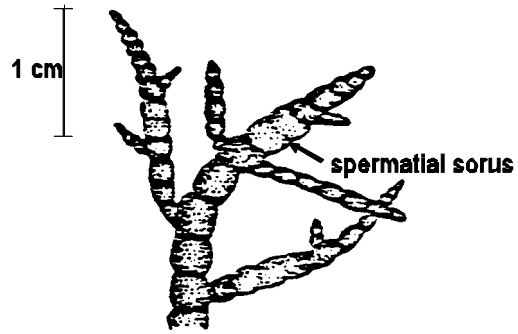

Figure 3. A portion of the male thallus showing spermatial sori. The sorus areas are generally slightly thicker and somewhat lighter in color. From USEPA (1987f).

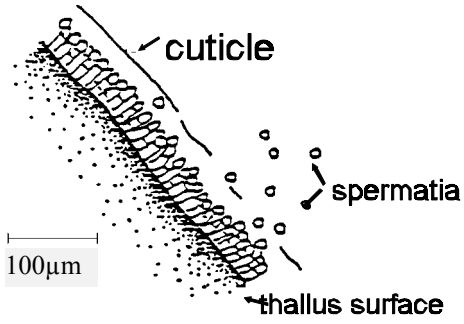

Figure 4. A magnified portion of a spermatial sorus. Note the rows of cells that protrude from the thallus surface. From USEPA (1987f).

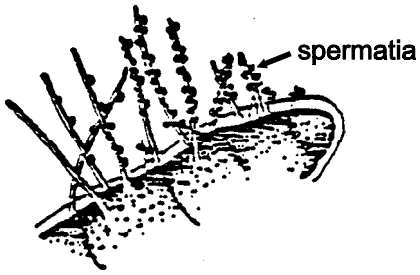

Figure 5. Apex of a branch on a mature female plant that was exposed to spermatia from a male plant. The sterile hairs and trichogynes are covered with spermatia. Note that few or no spermatia are attached to the older hairs (those more than l mm from the apex). From USEPA (1987f).

16.6.14.2.2 Alternately, 1-L flasks containing seawater can be autoclaved. Sterilization is used to prevent microalgal contamination, and not to keep cultures bacteria free.

16.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

16.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

16.8 CALIBRATION AND STANDARDIZATION

16.8.1 See Section 4, Quality Assurance.

16.9 QUALITY CONTROL

16.9.1 See Section 4, Quality Assurance.

16.10 TEST PROCEDURES

16.10.1 TEST SOLUTIONS

16.10.1.1 Receiving Waters

16.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 100 mL, and 400 mL for chemical analysis, would require approximately 800 mL or more of sample per test.

16.10.1.2 Effluents

16.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and allows for testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

16.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%).

16.10.1.2.3 The volume of effluent required for the test using a 0.5 dilution series is approximately 1800 mL. Prepare enough test solution at each effluent concentration (approximately 800 mL) to provide 100 mL of test solution for each of four (minimum of three) replicate test chambers and 400 mL for chemical analyses and record data (Figure 6).

16.10.1.2.4 Effluents can be tested at 100%. A 100% concentration of effluent can be achieved if the salinity of the effluent is adjusted to 30‰ by adding the GP2 dry salt formulation described in Table 2.

16.10.1.2.5 Just prior to test initiation (approximately 1 h), the temperature of sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (25 ± 1°C) and maintained at the temperature during the addition of dilution water.

SITE: _____

COLLECTION DATE: _____

TEST DATE: _____

LOCATION	INITIAL SALINITY	FINAL SALINITY	SOURCE OF SALTS FOR[1] SALINITY ADJUSTMENT

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

COMMENTS:

Figure 6. Data form for the red macroalga, *Champia parvula*, sexual reproduction test. Receiving water summary sheet. From USEPA (1987f).

16.10.1.2.6 Effluent dilutions should be prepared for all replicated in each treatment in one beaker to minimize variability among the replicates. The test chambers are labeled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

16.10.1.3 Dilution Water

16.10.1.3.1. The formula for the enrichment for natural seawater is listed in Table 1. Both EDTA and trace metals have been omitted. This formula should be used for the 2-day exposure period, but it is not critical for the recovery period. Since natural seawater quality can vary among laboratories, a more complete nutrient medium (e.g., the addition of EDTA) may result in faster growth (and therefore faster cystocarp development) during the recovery period.

16.10.2 PREPARATION OF PLANTS FOR TEST

16.10.2.1 Once cultures are determined to be usable for toxicity testing (have trichogynes and sori with spermatia), plant cuttings should be prepared for the test, using fine-point forceps, with the plants in a little seawater in a petri dish. For female plants, five cuttings, severed 7-10 mm from the ends of the branch, should be prepared for each treatment chamber. Try to be consistent in the number of branch tips on each cutting. For male plants, one cutting, severed 2.0 to 3.0 cm from the end of the branch, is prepared for each test chamber. Prepare the female cuttings first, to minimize the chances of contaminating them with water containing spermatia from the male stock cultures.

16.10.3 START OF TEST

16.10.3.1 Tests should begin as soon as possible after sample collection, preferably within 24 h. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test, Subsection 8.5.4).

16.10.3.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solution should be adjusted to the test temperature (23 ± 1°C) and maintained at that temperature during the addition of dilution water.

16.10.3.3 Label the test chambers with a marking pen. Use of color coded tape to identify each treatment and replicate is helpful. A minimum of five effluent concentrations and a control are used for each effluent test Each treatment (including controls) should have four (minimum of three) replicates.

16.10.3.4 Randomize the position of test chambers at the beginning of the test.

16.10.3.5 Prepare test solutions and add to the test chambers.

16.10.3.6 Add five female branches and one male branch to each test chamber. The toxicant must be present before the male plant is added.

16.10.3.7 Gently hand swirl the chambers twice a day, or shake continuously at 100 rpm on a rotary shaker.

16.10.3.8 If desired, the media can be changed after 24 h.

16.10.4 LIGHT, PHOTOPERIOD, SALINITY, AND TEMPERATURE

16.10.4.1 The light quality and intensity should be at 75 $\mu E/m^2/s$, or 500 foot candles (ft-c) with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 23 ± 1°C. The test salinity should be in the range of 28 to 32‰. The salinity should vary by no more than ± 2‰ among the chambers

on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

16.10.5 DISSOLVED OXYGEN (DO) CONCENTRATION

16.10.5.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentrations should be measured on new solutions at the start of the test (Day 0) and should be measured before renewal of the test solution after 24 h. The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests) If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1mL KIMAX® serological pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not occur.

16.10.6 OBSERVATIONS DURING THE TEST

16.10.6.1 Routine Chemical and Physical Observations

16.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in one test chamber at each concentration and in the control.

16.10.6.1.2 Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously, observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in environmental chamber.

16.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

16.10.6.1.4 Record all the measurements on the data sheet.

16.10.6.2 Routine Biological Observations

16.10.6.2.1 Protect the red macroalga from unnecessary disturbance during the test by carrying out the daily test observations and solution renewals carefully.

16.10.7 TRANSFER OF PLANTS TO CONTROL WATER AFTER 48 H

16.10.7.1 Label the recovery vessels. These vessels can be almost any type of container or flask containing 100 to 200 mL of seawater and nutrients (see Tables 1 and 2). Smaller volumes can be used, but should be checked to make sure that adequate growth will occur without having to change the medium.

16.10.7.2 With forceps, gently remove the female branches from test chambers and place into recovery bottles. Add aeration tubes and foam stoppers.

16.10.7.3 Place the vessels under cool white light (at the same irradiance as the stock cultures) and aerate for the 5-7 day recovery period. If a shaker is used, do not aerate the solutions (this will enhance the water motion).

16.10.8 TERMINATION OF THE TEST

16.10.8.1 At the end of the recovery period, count the number of cystocarps (Figures 7, 8, and 9) per female and record the data (Figure 10). Cystocarps may be counted by placing females between the inverted halves of a polystyrene petri dish or other suitable containers with a small amount of seawater (to hold the entire plant in one focal plane). Cystocarps can be easily counted under a stereomicroscope, and are distinguished from young branches because they possess an apical opening for spore release (ostiole) and darkly pigmented spores.

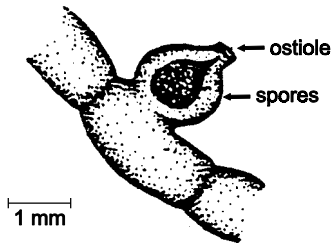

Figure 7. A mature cystocarp. In the controls and lower effluent concentrations, cystocarps often occur in clusters of 10 or 12. From USEPA (1987f).

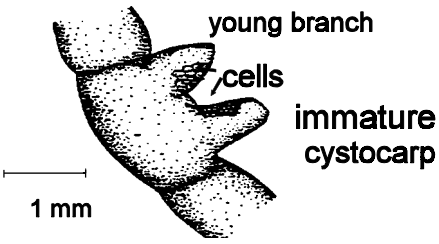

Figure 8. Comparison of a very young branch and an immature cystocarp. Both can have sterile hairs. Trichogynes might or might not be present on a young branch, but are never present on an immature cystocarp. Young branches are more pointed at the apex and are made up of larger cells than immature cystocarps, and never have ostioles. From USEPA (1987f).

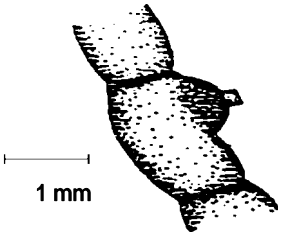

Figure 9. An aborted cystocarp. A new branch will eventually develop at the apex. From USEPA (1987f).

16.10.8.2 One advantage of this test procedure is that if there is uncertainty about the identification of an immature cystocarp, it is necessary only to aerate the plants a little longer in the recovery bottles. Within 24 to 48 h, the presumed cystocarp will either look more like a mature cystocarp or a young branch, or will have changed very little, if at all (i.e., an aborted cystocarp). No new cystocarps will form since the males have been removed, and the plants will only get larger. Occasionally, cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and, often, by the formation of a new branch at the apex.

16.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

16.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

16.12 ACCEPTABILITY OF TEST RESULTS

16.12.1 The test is acceptable if (1) control survival equals or exceeds 80% and (2) control plants average 10 or more cystocarps per plant.

16.12.2 If plants fragment in the controls or lower exposure concentrations, it may be an indication that they are under stress.

16.13 DATA ANALYSIS

16.13.1 GENERAL

16.13.1.1 Tabulate and summarize the data. A sample set of reproduction data is listed in Table 4.

16.13.1.2 The endpoints of the red macroalga, *Champia parvula*, toxicity test are based on the adverse effects on sexual reproduction as the mean number of cystocarps. The LC50, the IC25, and the IC50 are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). NOEC and LOEC values are obtained using a hypothesis testing approach, such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the IC25 and IC50. See the Appendices for examples of the manual computations, program listing, and example of data input and program output.

16.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

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							
OVERALL MEAN							

Temperature _____

Salinity _____

Light _____

Source of Dilution Water _____

Figure 10. Data form for the red macroalga, *Champia parvula*, sexual reproduction test. Cystocarp data sheet. From USEPA (1987f).

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION TEST WITH EFFLUENTS AND RECEIVING WATERS

CAUTION: This method is not listed at 40 CFR Part 136 for nationwide use.

1. Test type:	Static, non-renewal
2. Salinity:	30‰ (± 2‰ of the selected test salinity)
3. Temperature:	23 ± 1°C
4. Light quality:	Cool-white fluorescent lights
5. Light intensity:	75 µE/m^2/s (500 ft-c)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	200 mL polystyrene cups, or 250 mL Erlenmeyer flasks
8. Test solution volume:	100 mL (minimum)
9. No. organisms per test chamber:	5 female branch tips and 1 male plant
10. No. replicate per concentration:	4 (minimum of 3)
11. No. organisms per concentrations:	24 (minimum of 18)
12. Dilution water:	30‰ salinity natural seawater, or a combination of 50% of 30‰ salinity natural seawater and 50% of 30‰ salinity GP2 artificial seawater (see Section 7, Dilution Water)
13. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water or minimum of 5 and a control
14. Dilution factor:	Effluents: ≥ 0.5 Receiving waters: None or≥ 0.5

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION TEST WITH EFFLUENTS AND RECEIVING WATERS (CONTINUED)

15. Test duration:	2 day exposure to effluent, followed by 5 to 7-day recovery period in control medium for cystocarp development
16. Endpoints:	Reduction in cystocarp production compared to controls
17. Test acceptability criteria	80% or greater survival, and an average of 10 cystocarps per plant in controls
18. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sampling Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)
19. Sample volume required:	2 L per test

16.13.2 EXAMPLE OF ANALYSIS OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION DATA

16.13.2.1 Formal statistical analysis of the data is outlined in Figure 11. The response used in the analysis is the mean number of cystocarps per replicate chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint and the IC50 endpoint. Concentrations that have exhibited no sexual reproduction (less than 5% of controls) are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the IC endpoints.

16.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test. The assumptions of Dunnett's Procedures, normality and homogeneity of variance are formally tested. The test for normality is the Shapiro-Wilk's Test and Bartlett's Test is used to test for homogeneity of variance. Tests for normality and homogeneity of variance are included in Appendix B. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are determined by the parametric test.

TABLE 4. DATA FROM THE RED MACROALGA, *CHAMPIA PARVULA*, EFFLUENT TOXICITY TEST. CYSTOCARP COUNTS FOR INDIVIDUAL PLANTS AND MEAN COUNT PER TEST CHAMBER FOR EACH EFFLUENT CONCENTRATION[1]

Effluent Concentration (%)	Replicate Test Chamber	Plant 1	2	3	4	5	Mean Cystocarp Count
Control	A	19	20	24	7	18	17.60
	B	19	12	21	11	23	17.20
	C	17	25	18	20	16	19.20
0.8	A	10	16	11	12	11	12.00
	B	12	10	6	9	10	9.40
	C	12	9	9	13	8	10.20
1.3	A	10	0	3	5	4	4.40
	B	6	4	4	8	4	5.20
	C	4	4	2	6	4	4.00
2.2	A	1	2	5	4	0	2.40
	B	7	9	9	4	6	7.00
	C	3	2	2	0	0	1.40
3.6	A	2	1	1	5	0	1.80
	B	3	4	6	4	2	3.80
	C	0	4	3	1	3	2.20
6.0	A	1	0	0	0	0	0.20
	B	1	2	1	0	0	0.80
	C	0	4	3	1	3	2.20
10.0	A	0	0	0	0	-	0.00
	B	1	0	0	0	0	0.20
	C	2	1	0	0	0	0.60

[1] Data provided by the ERL-N, USEPA, Narragansett, RI.

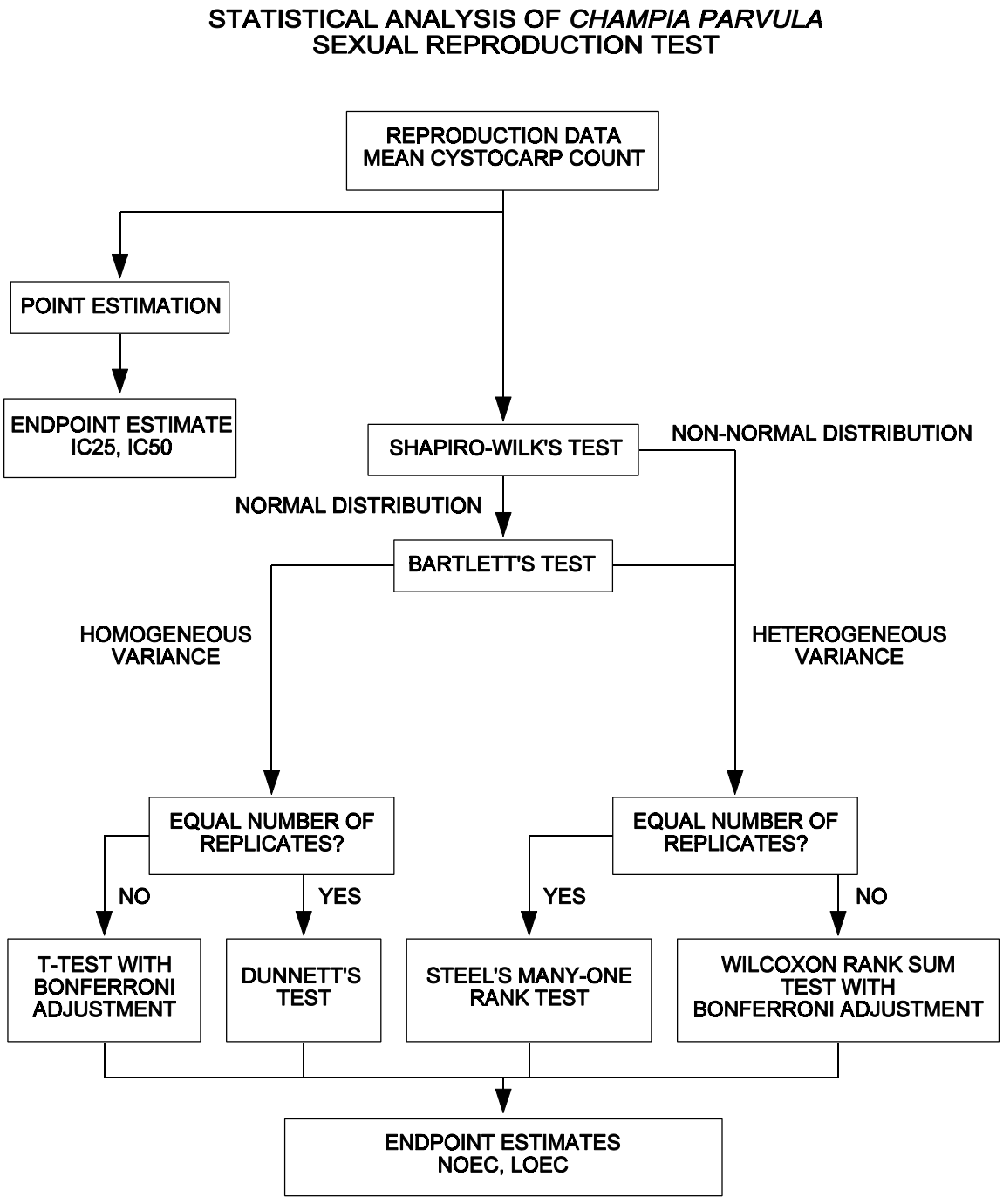

Figure 11. Flowchart for statistical analysis of the red macroalga, *Champia parvula*, data

6.13.2.3 If unequal numbers of replicates occur among the concentration levels tested there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

16.13.2.4 Example of Analysis of Reproduction Data

16.13.2.4.1 In this example, the data, mean and standard deviation of the observations at each concentration including the control are listed in Table 5. The data are plotted in Figure 12. As can be seen from the data in the table, mean reproduction per chamber in the 10% effluent concentration is less than 5% of the control. Therefore the 10% effluent concentration is not included in the subsequent analysis.

TABLE 5. RED MACROALGA, *CHAMPIA PARVULA*, SEXUAL REPRODUCTION DATA

		Effluent Concentration (%)					
Replicate	Control	0.8	1.3	2.2	3.6	6.0	10.0
A	17.60	12.00	4.40	2.40	1.80	0.20	0.00
B	17.20	9.40	5.20	7.00	3.80	0.80	0.20
C	19.20	10.20	4.00	1.40	2.20	2.20	0.60
Mean($\bar{Y}_i$)	18.00	10.53	4.53	3.60	2.60	1.07	0.27
S_i^2	1.12	1.77	0.37	8.92	1.12	1.05	0.09
i	1	2	3	4	5	6	7

16.13.2.5 Test for Normality

16.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all the observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

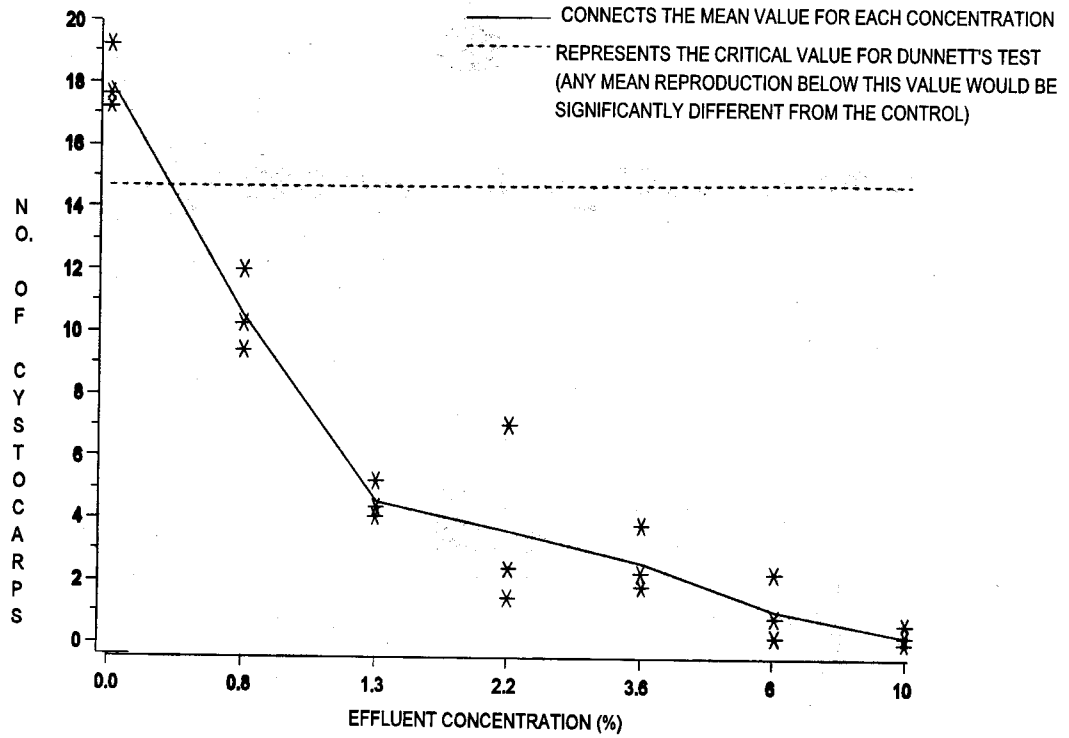

Figure 12. Plot of the number of cystocarps per plant.

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Effluent Concentration (%)				
Replicate	Control	0.8	1.3	2.2	3.6	6.0
A	-0.40	1.47	-0.13	-1.20	-0.80	-0.87
B	-0.80	-1.13	0.67	3.40	1.20	-0.27
C	1.20	-0.33	-0.53	-2.20	-0.40	1.13

16.13.2.5.2 Calculate the denominator, D, of the test statistic:

$$D = \sum_{i=1}^{n} (x_i - \overline{X})^2$$

Where: X_i = the ith centered observation

x = the overall mean of the centered observations

n = the total number of centered observations.

16.13.2.5.3 For this set of data, n = 18

$$\overline{X} = \frac{1}{8}(0.01) = 0.00$$

$$D = 28.7201$$

16.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq \ldots \leq X^{(n)}$$

Where $X^{(i)}$ is the ith ordered observation. These ordered observations are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

I	$X^{(i)}$	i	$X^{(i)}$
1	-2.20	10	-0.33
2	-1.20	11	-0.27
3	-1.13	12	-0.13
4	-0.87	13	0.67
5	-0.80	14	1.13
6	-0.80	15	1.20
7	-0.53	16	1.20
8	-0.40	17	1.47
9	-0.40	18	3.40

16.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots, a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 and k = 9. The a_i values are listed in Table 8.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	5.60	$X^{(18)} - X^{(1)}$
2	0.3253	2.67	$X^{(17)} - X^{(2)}$
3	0.2553	2.33	$X^{(17)} - X^{(3)}$
4	0.2027	2.07	$X^{(15)} - X^{(4)}$
5	0.1587	1.93	$X^{(14)} - X^{(5)}$
6	0.1197	1.47	$X^{(13)} - X^{(6)}$
7	0.0837	0.40	$X^{(12)} - X^{(7)}$
8	0.0496	0.13	$X^{(11)} - X^{(8)}$
9	0.0163	0.07	$X^{(10)} - X^{(9)}$

16.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}[\sum_{i=1}^{k} a_i(X^{(n-i+1)} - X^{(i)}]^2$$

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 8. For the data,

$$W = \frac{1}{28.7201}(5.1425)^2 = 0.921$$

16.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 16.3.2.5.6 with the critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For this example, the critical value at a significance level of 0.01 and 18 observations (n) is 0.858. Since W = 0.921 is greater than the critical value, conclude of the test is that the data are normally distributed.

16.13.2.6 Test for Homogeneity of Variance

16.13.2.6.1 The test used to examine whether the variation in mean cystocarp production is the same across all effluent concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ \ln\overline{S}^2 - \sum_{i=1}^{p} V_i \ln S_i^2]}{C}$$

Where: V_i = degrees of freedom for each effluent concentration and control, $V_i = (n_i - 1)$

p = number of levels of effluent concentration including the control

n_i = the number of replicates for concentration i

ln = $\log_e$

i = 1, 2, ..., p where p is the number of concentrations

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

16.13.2.6.2 For the data in this example (See Table 5) all effluent concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

16.13.2.6.3 Bartlett's statistic is therefore:

$$B = [(12)\ln(2.3917) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.194$$

$$= [12(0.8720) - 2(1n(1.12)+\ln(1.77)+...+\ln(1.05))]/1.1944$$

$$= (10.4640 - 4.0809)/1.1944$$

$$= 5.34$$

16.13.2.6.4 B is approximately distributed as chi-square with p - 1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with five degrees of freedom, is 15.09. Since B = 5.34 is less than the critical value of 15.09, conclude that the variances are not different.

16.13.2.7 Dunnett's Procedure

16.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p - 1)
Within	N - p	SSW	S_W^2 = SSW/(N - p)
Total	N - 1	SST	

Where: p = number effluent concentrations including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$$SSW = SST - SSB \qquad \text{Within Sum of Squares}$$

G = the grand total of all sample observations,

$$G = \sum_{i=1}^{p} T_i$$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the mean (across plants) number of cystocarps for effluent concentration i in test chamber j)

16.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$

$N = 18$
$T_1 = Y_{11} + Y_{12} + Y_{13} = 17.6 + 17.2 + 19.2 = 54$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 12.0 + 9.4 + 10.2 = 31.6$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 4.4 + 5.2 + 4.0 = 13.6$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.4 + 7.0 + 1.4 = 10.8$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 1.8 + 3.8 + 2.2 = 7.8$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 0.2 + 0.8 + 2.2 = 3.2$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 121.0$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$$= \frac{1}{3}(4287.24) - \frac{(121.0)^2}{18} = 615.69$$

$$SST = \sum_{i=1}^{p}\sum_{j=1}^{n_j} Y_{ij}^2 - G^2/N$$

$$= 1457.8 - \frac{(121.0)^2}{18} = 644.41$$

$$SSW = SST - SSB$$

$$= 644.41 - 615.69 = 28.72$$

S_B^2 = SSB/(p-1) = 615.69/(6-1) = 123.14

S_W^2 = SSW/(N-p) = 28.72/(18-6) = 2.39

16.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	615.69	123.14
Within	12	28.72	2.39
Total	17	644.41	

16.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_W\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean number of cystocarps for effluent concentration i

$\bar{Y}_1$ = mean number of cystocarps for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

16.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 0.8% concentration with the control the calculation is as follows:

$$t_2 = \frac{(18 - 10.53)}{[1.55\sqrt{(1/3) + (1/3)}]} = 5.9$$

16.13.2.7.6 Since the purpose of this test is to detect a significant reduction in cystocarp production, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix C. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. Mean cystocarp production for concentration i is considered significantly less control if t_i is greater than the critical value. Therefore, mean cystocarp productions for all effluent concentrations in this example have significantly lower cystocarp production than the control. Hence the NOEC is 0.8% and the LOEC is 0.8%.

TABLE 11. CALCULATED T VALUES

Effluent Concentration(%)	i	t_i
0.8	2	5.90
1.3	3	10.64
2.2	4	11.38
3.6	5	12.17
6.0	6	13.38

16.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = dS_W\sqrt{(1/n_1)+(1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration
(this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

16.13.2.7.8 In this example,

$$MSD = 2.50(1.55)\sqrt{(1/3)+(1/3)}$$

$$= 2.50\ (1.55)(.8165)$$

$$= 3.16$$

16.13.2.7.9 Therefore, for this set of data, the minimum difference that can be detected as statistically significant is 3.16 cystocarps.

16.13.2.7.10 This represents a 17.6% reduction in cystocarp production from the control.

16.13.2.8 Calculation of the ICp

16.13.2.8.1 The sexual reproduction data in Table 5 are utilized in this example. Table 12 contains the mean number of cystocarps for each effluent concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp. Refer to Figure 12 for a plot of the response curve.

TABLE 12. RED MACROALGA, *CHAMPIA PARVULA*, MEAN NUMBER OF CYSTOCARPS

Effluent Conc. (%)	i	Response Means $\bar{Y}_i$ (mg)	Smoothed Means M_i (mg)
Control	1	18.00	18.00
0.8	2	10.53	10.53
1.3	3	4.53	4.53
2.2	4	3.60	3.60
3.6	5	2.60	2.60
6.0	6	1.07	1.07
10.0	7	0.27	0.27

16.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in mean number of cystocarps, compared to the controls, would result in a mean number of 13.50 cystocarps, where $M_1(1-p/100) = 18.00(1-25/100)$. A 50% reduction in mean number of cystocarps, compared to the controls, would result in a mean number of 9.00 cystocarps. Examining the means and their associated concentrations (Table 12), the response, 13.50, is bracketed by $C_1 = 0.0\%$ effluent and $C_2 = 0.8\%$ effluent. The response, 9.00, is bracketed by $C_2 = 0.8\%$ effluent and $C_3 = 1.3\%$ effluent.

16.13.2.8.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 0.0 + [18.00(1 - 25/100) - 18.00] \frac{(0.8 - 0.0)}{(10.53 - 18.00)}$$

$$= 0.5\%.$$

16.13.2.8.4 Using the equation from Section 4.2 from Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 0.8 + [18.00(1 - 50/100) - 10.53] \frac{(1.3 - 0.8)}{(4.53 - 10.53)}$$

$$= 0.9\%$$

16.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 0.4821%. The empirical 95.0% confidence interval for the true mean was 0.4013% to 0.6075%. The computer program output for the IC25 for this data set is shown in Figure 13.

16.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 0.9278%. The empirical 95.0% confidence interval for the true mean was 0.7893% and 1.0576%. The computer program output for the IC50 for this data set is shown in Figure 14.

16.14 PRECISION AND ACCURACY

16.14.1 PRECISION

16.14.1.1 Single-Laboratory Precision

16.14.1.1.1 The single-laboratory precision data from six tests with copper sulfate (Cu) and six tests with sodium dodecyl sulfate (SDS) are listed in Tables 13-16. The NOECs with Cu differed by only one concentration interval (factor of two), showing good precision. The precision of the first four tests with SDS was somewhat obscured by the choice of toxicant concentrations, but appeared similar to that of Cu in the last two tests. The IC25 and IC50 are indicated in Tables 13-16. The coefficient of variation, based on the IC25 for these two reference toxicants in natural seawater and a mixture of natural seawater and GP2, ranged from 59.6% to 69.0%, and for the IC50, ranged from 22.9% to 43.7%.

16.14.1.1.2 EPA evaluated single-laboratory (within-laboratory) precision of the Red Macroalga, *Champia parvula*, Reproduction Test using a database of routine reference toxicant test results from two laboratories (USEPA, 2000b). The database consisted of 23 reference toxicant tests conducted in 2 laboratories using reference toxicants including: copper and sodium dodecyl sulfate. The within-laboratory CVs calculated for routine reference toxicant tests at these 2 laboratories were 58% and 59% for the IC25 reproduction endpoint.

16.14.1.2 Multilaboratory Precision

16.14.1.2.1 The multilaboratory precision of the test has not yet been determined.

16.14.2 ACCURACY

16.14.2.1 The accuracy of toxicity tests cannot be determined.

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	.8	1.3	2.2	3.6	6	10
Response 1	19	10	10	1	2	1	0
Response 2	20	16	0	2	1	0	0
Response 3	24	11	3	5	1	0	0
Response 4	7	12	5	4	5	0	0
Response 5	18	11	4	0	0	0	1
Response 6	19	12	6	7	3	1	0
Response 7	12	10	4	9	4	2	0
Response 8	21	6	4	9	6	1	0
Response 9	11	9	8	4	4	0	0
Response 10	23	10	4	6	2	0	2
Response 11	17	12	4	3	0	0	1
Response 12	25	9	4	2	4	4	0
Response 13	18	9	2	2	3	3	0
Response 14	20	13	6	0	1	1	0
Response 15	16	8	4	0	3	3	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: effluent Test Start Date: Test Ending Date:
Test Species: RED MACROALGA, Champia parvula
Test Duration: DATA FILE: champia.icp OUTPUT FILE: champia.i25

Conc. ID	Number Replicates	Concentration %	Response Means	Standard. Dev.	Pooled Response Means
1	15	0.000	18.000	4.928	18.000
2	15	0.800	10.533	2.356	10.533
3	15	1.300	4.533	2.356	4.533
4	15	2.200	3.600	3.066	3.600
5	15	3.600	2.600	1.805	2.600
6	15	6.000	1.067	1.335	1.067
7	15	10.000	0.267	0.594	0.267

The Linear Interpolation Estimate: 0.4821 Entered P Value: 25

Number of Resamplings: 80 The Bootstrap Estimates Mean: 0.4947 Standard Deviation: 0.0616
Original Confidence Limits: Lower: 0.4013 Upper: 0.6075
Resampling time in Seconds: 3.68 Random Seed: 703617166

Figure 13. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6	7
Conc. Tested	0	.8	1.3	2.2	3.6	6	10
Response 1	19	10	10	1	2	1	0
Response 2	20	16	0	2	1	0	0
Response 3	24	11	3	5	1	0	0
Response 4	7	12	5	4	5	0	0
Response 5	18	11	4	0	0	0	1
Response 6	19	12	6	7	3	1	0
Response 7	12	10	4	9	4	2	0
Response 8	21	6	4	9	6	1	0
Response 9	11	9	8	4	4	0	0
Response 10	23	10	4	6	2	0	2
Response 11	17	12	4	3	0	0	1
Response 12	25	9	4	2	4	4	0
Response 13	18	9	2	2	3	3	0
Response 14	20	13	6	0	1	1	0
Response 15	16	8	4	0	3	3	0

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: effluent Test Start Date: Test Ending Date:
Test Species: RED MACROALGA, Champia parvula
Test Duration: DATA FILE: champia.icp OUTPUT FILE: champia.i50

Conc. ID	Number Replicates	Concentration %	Response Means	Standard. Dev.	Pooled Response Means
1	15	0.000	18.000	4.928	18.000
2	15	0.800	10.533	2.356	10.533
3	15	1.300	4.533	2.356	4.533
4	15	2.200	3.600	3.066	3.600
5	15	3.600	2.600	1.805	2.600
6	15	6.000	1.067	1.335	1.067
7	15	10.000	0.267	0.594	0.267

The Linear Interpolation Estimate: 0.9278 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 0.9263 Standard Deviation: 0.0745
Original Confidence Limits: Lower: 0.7893 Upper: 1.0576
Resampling time in Seconds: 3.63 Random Seed: -1255453122

Figure 14. ICPIN program output for the IC50.

TABLE 13. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST PERFORMED IN A 50/50 MIXTURE OF NATURAL SEAWATER AND GP2 ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS CULTURED IN NATURAL SEAWATER. THE REFERENCE TOXICANT USED WAS COPPER (CU) SULFATE[1,2,3,4,5]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	1.0	1.67	2.37
2	1.0	1.50	1.99
3	1.0	0.69	1.53
4	1.0	0.98	1.78
5	0.5	0.38	0.76
6	0.5	0.38	0.75
n:	6	6	6
Mean:	NA	0.93	1.5
CV(%):	NA	59.6	43.7

[1] Data from USEPA (1991a).
[2] Tests performed by Glen Thursby and Mark Tagliabue, ERL-N, USEPA, Narragansett, RI. Tests were conducted at 22°C, in 50/50 GP2 and natural seawater at a salinity of 30‰.
[3] Copper concentrations were: 0.5, 1.0, 2.5, 5.0, 7.5, and 1.0 µg/L.
[4] NOEC Range: 0.5 - 1.0 µg/L (this represents a difference of one exposure concentration).
[5] For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 14. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST PERFORMED IN A 50/50 MIXTURE OF NATURAL SEAWATER AND GP2 ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS CULTURED IN NATURAL SEAWATER. THE REFERENCE TOXICANT USED WAS SODIUM DODECYL SULFATE (SDS)[1,2,3,4,5]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	< 0.80	0.6	0.3
2	0.48	0.7	0.6
3	< 0.48	0.4	0.2
4	< 0.48	0.2	0.4
5	0.26	0.2	0.5
6	0.09	0.1	0.3
7	0.16	0.2	0.3
8	0.09	0.1	0.2
9	< 0.29	0.3	0.4
n:	5	9	9
Mean:	NA	0.31	0.36
CV(%):	NA	69.0	37.0

1 Data from USEPA (1991a).

2 Tests performed by Glen Thursby and Mark Tagliabue, ERL-N, USEPA, Narragansett, RI. Tests were conducted at 22°C, in 50/50 GP2 and natural seawater at a salinity of 30‰.

3 SDS concentrations for Test 1 were: 0.8, 1.3, 2.2, 3.6, 6.0, and 10.0 mg/L. SDS concentrations for Tests 2, 3, and 4 were: 0.48, 0.8, 1.3, 2.2, 3.6, and 6.0 mg/L. SDS concentrations for Tests 5 and 6 were: 0.09, 0.16, 2.26, 0.43, 0.72, and 1.2 mg/L.

4 NOEC Range: 0.09 - 0.48 mg/L (this represents a difference of two exposure concentrations).

5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 15. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST IN NATURAL SEAWATER (30‰ SALINITY). THE REFERENCE TOXICANT USED WAS COPPER (CU) SULFATE[1,2,3]

	Cu (µg/L)		
Test	NOEC	IC25	IC50
1	1.00	2.62	4.02
2	0.50	0.71	1.66
3	0.50	2.83	3.55
4	0.50	0.99	4.15
n:	4	4	4
Mean:	NA	1.79	3.35
CV(%):	NA	61.09	34.45

1 Data from USEPA (1991a).
2 Copper concentrations were 0.5, 1.0, 2.5, 5.0, 7.5, and 10 µg/L. Concentrations of Cu were made from a 100 µg/mL $CuSO_4$ standard obtained from Inorganic Ventures, Inc., Brick, NJ.
3 Prepared by Steven Ward and Glen Thursby, Environmental Research Laboratory, USEPA, Narragansett, RI.

TABLE 16. SINGLE-LABORATORY PRECISION OF THE RED MACROALGA, *CHAMPIA PARVULA*, REPRODUCTION TEST IN NATURAL SEAWATER (30‰ SALINITY). THE REFERENCE TOXICANT USED WAS SODIUM DODECYL SULFATE (SDS)[1,2,3]

	SDS (mg/L)		
Test	NOEC	IC25	IC50
1	0.60	0.05	0.50
2	0.60	0.48	0.81
3	0.30	0.69	0.89
4	0.15	0.60	0.81
n:	4	4	4
Mean:	NA	0.46	0.75
CV(%):	NA	62.29	22.92

1 Data from USEPA (1991a).
2 SDS concentrations were 0.0375, 0.075, 0.15, 0.03, 0.60, and 1.20 mg/L. Concentrations of SDS were made from a 44.64 ± 3.33 mg/mL standard obtained from the EMSL-USEPA, Cincinnati, OH.
3 Prepared by Steven Ward and Glen Thursby, Environmental Research Laboratory, USEPA, Narragansett, RI.

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