Development of short-term exposure tests for marine macroalgae for use in effluent testing

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DEVELOPMENT OF SHORT-TERM EXPOSURE TESTS FOR MARINE MACROALGAE FOR USE IN EFFLUENT TESTING

by

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OVERVIEW

A relatively fast, simple and inexpensive toxicity test method has been developed for the marine red macroalga Champia parvula, to assess chronic effects of pollutants to marine macroalgae. The method has been used to generate toxicity data for water quality criteria. The test method has previously been evaluated with heavy metals and cyanide, arsenate and arsenite, as well as ten different organic compounds. This test method showed that sexual reproduction is the best endpoint to use for Champia. If sexual reproduction is used as the sole endpoint, then plants should only have to be exposed to toxicants for a few days (long enough to show any effects on fertilization). The previous toxicity test with Champia lasted two weeks, however, a modification has been developed in which females and males are exposed together to a toxicant for only two days. The procedure has been used successfully with single compounds and a variety of complex effluents.

Initial steps were also taken in the development of a similar short-term test with the brown alga, Laminaria saccharina. Laminaria represents another phylum of algae than Champia, and has the additional advantage of being both economically and ecologically important. Sexual reproduction in Laminaria has already been shown to be sensitive to petroleum products. For the current study, the feasibility of using Laminaria as a routine toxicity test species was verified. The large adult sporophyte of Laminaria is difficult to maintain in the laboratory. However, the male and female gametophytes are microscopic and are easily cultured.

The Office of Water's Permits Division of the Environmental Protection Agency needs toxicity test procedures for marine and

estuarine species. The procedures are needed to characterize and quantify the toxicity of effluent discharges by National Pollutant Discharge Elimination System permittees. The tests must yield chronic data in a relatively short time period (7 days or less). The data must be both scientifically sound and legally defensible. At the same time, it is necessary for the methods to be simple and cost efficient (requiring standard hardware and laboratory facilities). The test species should be readily available, and testing should be practical for both on-site and off-site. All of these requirments are met by the test methods developed for the marine algae, Champia parvula and Laminaria saccharina.

This report covers work completed during the time period from November 1, 1984 through October 31, 1986. The specific objectives of the cooperative agreement were:

I. Champia parvula

A. Write final guidance manual for conducting short-term chronic tests.

B. Compare data from above test procedure with that from the existing two-week test.

C. Test short-term test in field and laboratory with complex effluents.

II. Laminaria saccharina

A. Test feasibility of Laminaria saccharina as a routine toxicity test species for short-term tests.

B. Compare the sensitivity of Laminaria with that of Champia.

The techniques developed during this cooperative agreement were presented at three effluent monitoring workshops. The Champia technique was presented at EPA's Narragansett, Rhode Island laboratory in October, 1985, and at EPA's Gulf Breeze, Florida laboratory in February, 1986. The Laminaria technique was presented at the workshop held in Newport, Oregon, in October, 1986.

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PART I

GUIDANCE MANUAL FOR CONDUCTING SEXUAL REPRODUCTION TEST WITH THE MARINE MACROALGA *CHAMPIA PARVULA* FOR USE IN TESTING COMPLEX EFFLUENTS

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INTRODUCTION

Seaweeds have been considered less useful for toxicity testing than microalgae (Jensen, 1984), and microalgae are often considered less sensitive than aquatic animals (Kenaga and Moolenaar, 1976; Kenaga, 1982). Therefore, one could easily come to the erroneous conclusion that toxicity testing with seaweeds is not necessary. As recently as 1983 the statement was made that "seaweeds seem to be rather insensitive to many chemicals and will probably survive pollution better than many other organisms in the marine environment" (Jensen, 1984).

A two-week toxicity test method has already been developed for the macroalga, Champia parvula, to assess chronic effects of pollutants to marine seaweeds (Steele and Thursby, 1983). The test has previously been evaluated with heavy metals (Steele and Thursby, 1983), arsenite and arsenate (Thursby and Steele, 1984), and ten different organic compounds (Thursby, et al., 1985). This test method shows that sexual reproduction is generally the most sensitive and practical endpoint to use for Champia.

Pollution assessments with macroalgae must take reproduction into consideration if an accurate picture of the potential harm is to be drawn. Previous conclusions about seaweed sensitivity were based largely on growth as the endpoint. The ability to measure the sensitivity of seaweeds to toxicants increases when sexual reproduction is used as an endpoint; and can be greater than many aquatic animals that have been tested. This has been shown for Champia parvula (see above references) as well as for the brown macroalgae Fucus edentatus and Laminaria saccharina (Steele and Hanisak, 1978).

The above toxicity test with Champia parvula is a two-week growth and reproduction study and requires that the cultures remain unialgal.

This time period makes the test unaccepatable for testing complex effluents and their receiving waters. Fine-filtering, which would be necessary to remove unwanted microalgae, could change the character of the effluent or receiving water. Any microalgae introduced with the effluent would compete with Champia for light and nutrients, thus influencing Champia's growth rate. However, if sexual reproduction is used as the sole endpoint, then plants only need to be exposed for a few days (long enough to show effects on fertilization). Any effect of other organisms on the growth rate of Champia would not be serious since interest would only be in whether sexual reproduction had taken place.

Sexual reproduction was selected as the endpoint for effluent testing for several reasons. It had previously proven to be a sensitive endpoint from the two-week toxicity test procedure using single compounds. A sexual reproduction test for toxicity could be short enough to fit the time-constraints for tests used in the effluent program. Finally, Champia is an annual plant and inhibition or absence of sexual reproduction reduces or eliminates the next stage in its life history. Total absence of cystocarp formation is the easiest endpoint to interpret as far as field populations are concerned. In most of the red algae each fertilization results in the formation of a new life history stage, the carposporophyte, "parasitic" on the female and housed within the cystocarp. Each carposporophyte is capable of producing many spores (perhaps a hundred or more in the case of Champia). This characteristic makes it difficult to interpret the biological significance of a statistical decrease in the number of cystocarps or an arbitrary percent decrease such as 50%. Absence of reproduction leaves no doubt about its biological significance.

BACKGROUND

This paper describes a method which uses sexual reproduction, after a short-term exposure to effluents, to estimate chronic toxicity. In brief, the method consists of exposing males and females to effluents or receiving waters for two days, followed by a 5- to 7-day recovery period in control medium. The recovery period allows time for any cystocarps to mature. At the end of the recovery period the number of cystocarps per plant are counted. The goal for Champia within the effluent program is to use absence of sexual reproduction as the endpoint. Statistical differences (or other "cut-offs"), although more difficult to interpret ecologically, can also be included to more easily make comparisons with other marine species in the program. In addition, the reporting of concentrations that cause the total absence of sexual reproduction and statistical differences will give some idea of the steepness of the dose response curve.

The method described here has been used for both single compounds and complex effluents. Tests have been conducted on-site in a mobile laboratory and at the EPA's Environmentl Research Laboratory, Narragansett, RI. Several different types of effluents have been tested. These included one from a pulp mill; two industrial sites that discharge effluents containing heavy metals; five industrial sites discharging organically contaminated effluents, including pesticides and dyes; and 17 different sewage effluents. In addition several receiving waters have also been tested. In all, more than 80 tests have been performed. When basing the test endpoint on the absence of reproduction, the pulp mill effluent had its effect between 1.0 and 2.5 % effluent; the heavy metal effluents had a range of effect of 0.054 to 0.50 % effluent;

organic effluents had a range of 3 to 20 % effluent (generally <5 %); and the sewage effluents ranged from 2.5 to 25 % effluent (generally < 10 %). Receiving water effects have been detected and ranged from little or no effect on sexual reproduction to total elimination of sexual reproduction.

Nine single compounds have been used to compare effects on sexual reproduction using the two-week test and the two-day exposure. Several different cut-off points for the endpoint of sexual reproduction from the two-day exposure were compared against the no sexual reproduction (NSR) endpoint from the two-week test (Thursby and Steele, 1986). From among these comparisons 5%-of-the-control (95% or greater decrease) gave the best estimate of the NSR effect for the two-week test (Table 1). Therefore, cystocarp counts at $\leq$ 5% of the control are considered zero for the short-term exposure test.

The sexual reproduction, two-day exposure test has been developed as a static, non-renewal test (although daily media changes are possible) for effluents and receiving waters. The method is easy and cost efficient to perform. Stock cultures are maintained in the laboratory with standard laboratory equipment, therefore, plant material can be available year-round. The test procedure is intended to be used to estimate chronic effects of complex effluents on marine macroalgae.

Table 1. Comparison of the short-term exposure test and the two-week test using single compounds. Values listed are the geometric means of the effect and no-effect concentrations in ug/L. Effect is defined as either $\leq$ 5% of the control (short-term exposure test) or no sexual reproduction (two-week test).

COMPOUND	SHORT-TERM (STE)	TWO-WEEK (TWT)	RATIO (STE/TWT)
Arsenite	232	139	1.67
Cadmium	>100[a]	77	>1.30
Copper	7.7	7.7	1.00
Silver	0.92	1.50	0.61
Benzene	73,600[b]	73,600	1.00
Isophorone	107,050	107,050	1.00
Pentachloroethane	10,170	10,170	1.00
Pentachlorophenol	465	465	1.00
Toxaphene	140	140	1.00

[a]STE was run in polystrene cups, in 125 Erlenmeyer flasks and in 500 mL, screw-capped flasks with 400 mL of medium. The results were always the same. The STE may not work for cadmium because it is a slow toxicant and two days is not enough time to see its full effect.

[b]STE was run in 400 mL of medium in 500 mL, screw-capped flasks. The STE did not compare well with the TWT when the cups were used. A larger volume may be necessary when working with highly volatile compounds.

MAINTENANCE OF STOCK CULTURES OF CHAMPIA PARVULA

Plants of Champia parvula (C. Agardh) Harvey (Rhodophyta) are bushy and 5 to 10 cm tall in the field. The main axis and branches are cylindrical, hollow and septate. Champia's life history is an alternation of isomorphic generations (Fig. 1). The clone presently used was isolated from Rhode Island waters in 1979. It is probably not essential to have a standard clone, however, some experience is required to isolate new clones from the field into unialgal culture. Unialgal stock cultures are necessary to maintain healthy, actively growing plants for use in testing. Vegetatively propagated plant material from the 1979 clone is available from the U.S. Environmental Protection Agency, South Ferry Road, Narragansett, RI 02882.

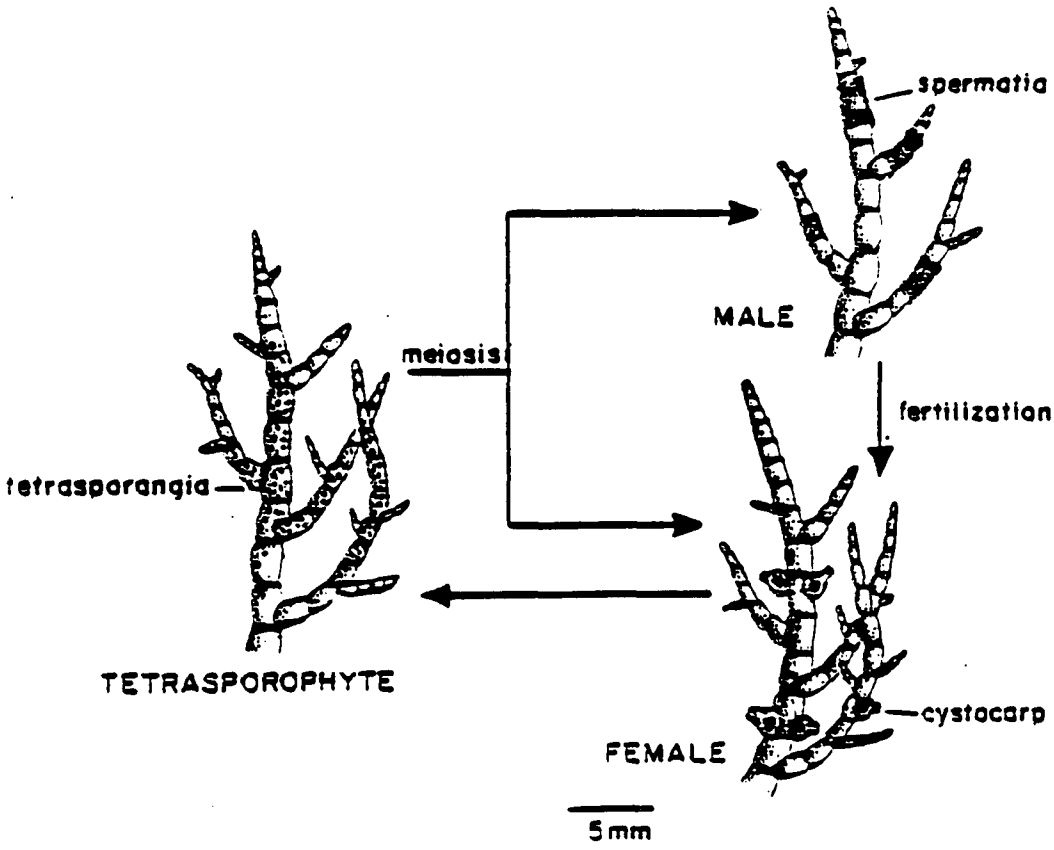

Figure 1. Life history of the marine red alga Champia parvula.

Male and female plants of Champia can be maintained easily in unialgal culture in the laboratory. New cultures can be started from excised branches, making it possible to maintain clonal material indefinitely. No special preconditioning is required to induce reproduction. Under the conditions listed below, male gametophytes produce spermatia continuously and females are always receptive. Thus, plant material can be available at any time for testing.

Laboratory cultures of Champia provide test plants of similar preconditioning. Unialgal stock cultures of both males and females are maintained in separate, aerated 1000 mL Erlenmeyer flasks containing 800 mL of the culture medium. The choice of this flask is one of preference rather than necessity. All culture glassware should be acid-stripped in 10 to 15 % HCl and rinsed in deionized water after washing. This is necessary since many detergents can leave a residue that is toxic to Champia. The culture medium is made from natural seawater to which additional nutrients are added (Table 2). The nutrients used with artificial seawater can also be used (see ARTIFICIAL SEAWATER). The seawater is autoclaved for 30 min at 15 psi. The culture flasks are capped with aluminum foil and autoclaved dry, for 10 min. Culture medium is made up by dispensing seawater into the sterile flasks and adding the appropriate nutrients from a sterile stock solution. Alternately, liter flasks could be autoclaved with the seawater already in them. Sterilization is used to prevent microalgal contamination, and not to keep cultures bacteria-free.

We recommend that several cultures of both males and females be maintained simultaneously to keep a constant supply of plant material available. Some cultures should be at different stages of development (i.e., with different amounts of tissue per flask). Initial stock

cultures should be started weekly with about twenty 1.0 cm branch tips. Cultures are gently aerated through sterile, cotton-plugged, disposable, polystyrene 1 mL pipets. Cultures are capped with foam plugs and aluminum foil and illuminated from the side with 75 uE m^{-2} s^{-1} of cool-white fluorescent light on a 16:8, light:dark cycle. The temperature is 22 to 24 °C and the salinity 28 to 30 °/₀₀. Media are changed once a week.

Table 2. Recipe for additional nutrients to be added to natural seawater for stock cultures and test medium. Both EDTA and trace metals have been omitted. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 minutes (the pH is adjusted to 2.0 prior to autoclaving to prevent precipitation).

COMPOUND	AMOUNT/LITER TEST MEDIUM CONC.	CONCENTRATED STOCK[a]
$NaNO_3$	9.35 mg	3.74 grams
$NaH_2PO_4 \cdot H_2O$	0.62 mg	0.25 grams
Iron[b]	2.6 ug	1.04 mg
Vitamins		
B12	0.06 ug	10 mL[c]
Biotin	0.06 ug	
Thiamine·HCl	12.5 ug	

[a]Use 0.25 mL/100mL (2.5 mL/L) for test medium concentrations and 10 mL/L for stock cultures. For test medium only, add 0.25 mL/100mL of a sodium bicarbonate solution. A stock solution of 60 mg/mL sodium bicarbonate is prepared by autocalving it as a dry powder and then dissolving it in sterile deionized water.

[b]Iron stock solution prepared by dissolving 1 g iron powder in 10 mL concentrated HCl and diluting to 1 liter with deionized water. Acceptable stock soultions could also be made with ferric or ferrous chloride.

[c]Vitamin stock solution autoclaved separately in 10 mL sub-samples. Each 10 mL contains 24 ug B12, 24 ug biotin and 5 mg thiamine·HCl. Adjust pH to ca 4.0 before autoclaving for 2 min.

About half of the plants should be discarded (or placed into another culture vessel) with each weekly medium change to reduce the amount of biomass as the plants grow. At the end of three weeks plants will be ready to use for testing. Readiness is defined as having enough plant material to perform at least one test. With this procedure, actively growing plants will be continuously available. The total number of cultures maintained will depend on the expected frequency of testing.

A stock culture should not be used as a source of test material if the plants appear to be stressed or undernourished. Under conditions of stress the tips of the branches will turn "pink" and the older tissue will generally be much paler. In addition, the sterile hairs of stressed plants will appear stubby, especially near the branch tips. The trichogynes of stressed female plants will also be stunted or absent. This can be evident even in plants that do not have any apparent color change. Under conditions of nutrient deficiency (resulting usually from too much plant material in the culture flask or too long since the last medium change) the entire plant will turn pale yellow. If the stress is severe enough the older tissues (main axes) or occasionally the branch tips will turn white (evidence of necrotic tissue). If cystocarps are present on females in the stock cultures, the plants are not suitable for testing (this usually happens as a result of contamination by males or water from male cultures).

ARTIFICIAL SEAWATER

Because salinity adjustments will be necessary in many complex effluent tests, an artificial seawater recipe that yields good growth of Champia is desirable. An artificial seawater would also make the test method more readily available to laboratories that do not have access to clean natural seawater. Some commercial preparations are toxic to Champia (presumably due to the presence of high concentrations of trace metals that are in the commercial grade of salts used in their preparation). We have had success with artificial seawater using GP2 (Spotte, et al., 1984). Plants grow and reproduce well and have the correct morphology in GP2, although they may be slightly smaller in diameter than plants grown in natural seawater. Plants require approximately two weeks to acclimate to the artificial seawater. The recipe for artificial seawater with GP2 is listed in Table 3.

A comparison between the sensitivity to toxicants in the GP2 medium with that in natural seawater has only been made for copper. Results suggest that plants grown and tested in GP2 are slightly less sensitive to copper than plants from natural seawater. This may be due to an acclimation to higher levels of heavy metals in the GP2 medium (from the reagent grade salts). However, comparisons with more compounds are needed before we can conclude that plants grown in GP2 are always less sensitive.

During the months of June, July and August the quality of the seawater that we normally use to culture and test Champia parvula is often poor. We have, however, been able to obtain excellent growth and reproduction from Champia during this period of time by mixing GP2 and natural seawater in a 50:50 ratio. The plants required no acclimation period to this mixture.

Table 3. Recipe for artificial seawater using GP2. The concentrations of the salts have been adjusted to give a final salinity of 30°/₀₀. The original recipe calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), then all of the 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 should be for at least 10 min for 1 liter batches and 20 min for 10 to 20 liter volumes (at standard temperature and pressure).

COMPOUND	GRAMS/LITER[a]
NaCl	21.03
Na_2SO_4	3.52
KCl	0.61
KBr	0.088
$Na_2B_4O_7 \cdot 10H_2O$	0.034
$MgCl_2 \cdot 6H_2O$	9.50
$CaCl_2 \cdot 2H_2O$	1.32
$SrCl_2 \cdot 6H_2O$	0.02
$NaHCO_3$[b]	0.17

[a]Generally made in 10 to 20L batches.

[b]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.

SALINITY ADJUSTMENTS

We have used natural seawater brine (made by evaporation to ca. 100 °/₀₀), GP2 brine (GP2 made to 3x strength), and concentrated GP2 salts added separately, to adjust brackish receiving waters to 30 °/₀₀. Salinity adjustments with dry salts are usually too cumbersome, especially for use in a mobile laboratory. If the initial salinity of the receiving water is >15 °/₀₀, then plants grown in natural seawater can be used with any of the above methods of salinity adjustment. However, if the initial salinity of the receiving water is <15 °/₀₀, then plants previously acclimated to artificial seawater are recommended. In either case, a control using clean natural seawater diluted with deionized water to the lowest salinity to be tested (then adjusted upwarded to 30 °/₀₀ with one of the techniques above) should be used. If the salinity range of the receiving waters to be tested from a given location is great, then it is advisable to make up diluted controls at several salinities.

Plants grown in artificial seawater have always done well when placed into natural seawater (i.e., no acclimation period required). However, plants grown in natural seawater do not always do well when placed into artificial seawater. This is true even for the two day exposure period.

PREPARATION OF PLANTS FOR A TEST

Stock cultures should be checked for their readiness for use in toxicity tests. 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 (Fig. 2). 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 (Fig. 3). Another way is to examine the males under a compound microscope. A mature sorus can be easily identified by looking at the edge of the thallus (Fig. 4). 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 attached to both the sterile hairs and the trichogynes (Fig. 5).

Once cultures are determined to be usable for toxicity testing, branch tips should be cut into their final size. For females, cut 7 to 10 mm branch tips, enough for 5 per treatment chamber (try to be consistent in the degree of branching; see Fig. 6). The cutting can be easily done with fine-point forceps with the plants in a little seawater in a petri dish. Repeat for males, except cut 1.5 to 2 cm branches and only one per treatment chamber. The males should visibly be producing spermatia (i.e. two or more spermatial sori present). Cut the females first to minimize the chances of contaminating them with water from the male stock cultures.

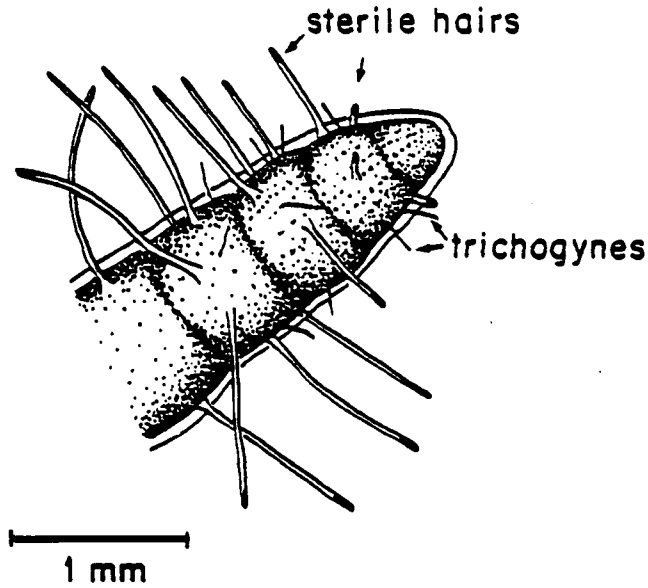

Figure 2. Apical tip of female branch of Champia parvula showing sterile hairs and trichogynes (reproductive hairs). Sterile hairs are wider and generally much longer than trichogynes. They also appear to be hollow except at their apex. Both types of hairs occur around the entire circumference of the thallus but are seen easiest at the "edges". Receptive trichogynes occur only near the branch tips.

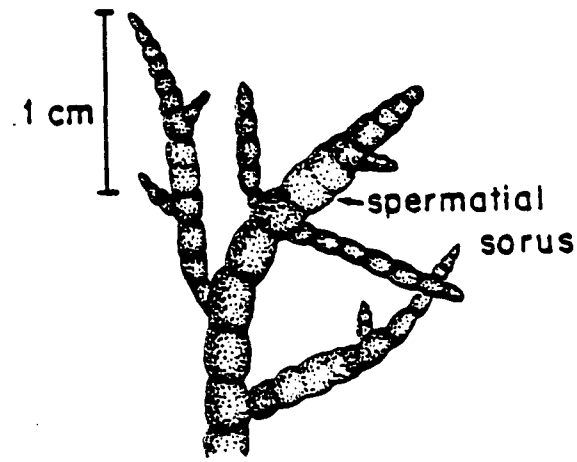

Figure 3. A portion of a male thallus of Champia parvula showing spermatial sori. The sorus areas are generally slightly thicker in diameter and a little lighter in color.

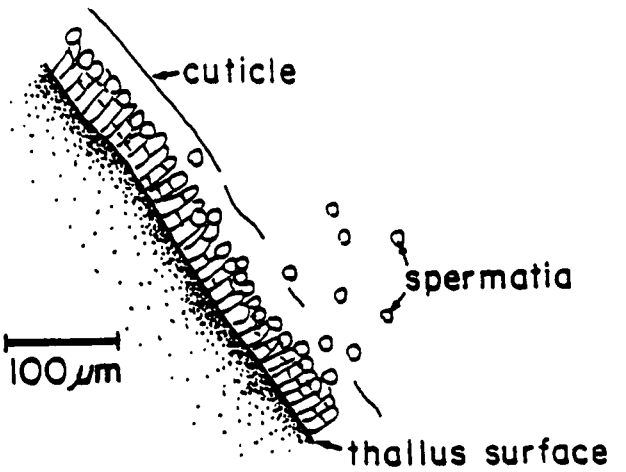

Figure 4. A close-up of a portion of a spermatial sorus, note the rows of cells that protrude from the thallus surface.

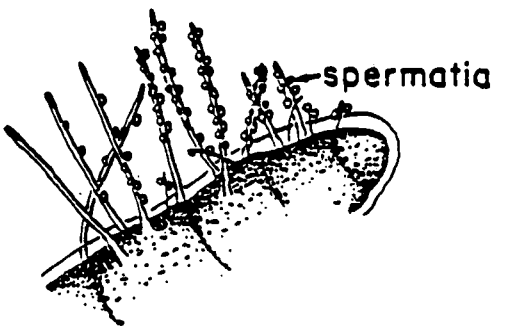

Figure 5. Apical tip of a female of Champia parvula that had been "dipped" in water from a male culture. The sterile and reproductive hairs are covered with spermatia. Note that older hairs (those more than about one mm back from the apex) have few to no spermatia attached to them.

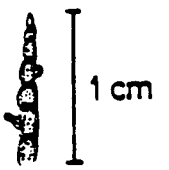

Figure 6. The size and degree of branching that is generally used for the female starting plants. Occasionally the branches will be longer near the tip, try to be consistent in the degree of branching since the receptive trichogynes are at the branch tips. Therefore, the more variation in the degree of branching, the more variation in the potential number of cystocarps per plant.

TEST CHAMBERS

Most testing to date has been performed with 100 mL in ca. 110 mL polypropylene cups with fitted polyethylene caps (Falcon®). These cups offer the advantage of being disposable, and their wide opening allows easy access to plants for transferring to recovery bottles. The use of these cups is out of preference rather than necessity. Successful tests have also been run in 125 mL Erlenmeyer, Pyrex® flasks and in 100 mL polystryene cups with plastic caps.

If glass test chambers are used, then they should be acid-stripped for ca. 10 min. in 10 to 15 % HCl and rinsed in deionized water after washing. This removes potentially toxic residues left by the detergent. If organic compounds have been previously tested in the glassware, then a rinse with acetone prior to washing is recommended.

TEST CONDITIONS

Temperature, Salinity, Light and Aeration

The test exposure duration is 2 days followed by a 5 to 7 day recovery period for the development of cystocarps. The exposure temperature should be between 22 and 24°C, and the test salinity should be between 28 and 32 °/$_{oo}$. For testing receiving waters, salinity will often be below the desired range and must be adjusted with artificial sea salts (see SALINITY ADJUSTMENTS). The photoperiod should be a 16h:8h, light:dark cycle of ca. 75 uE m^{-2} s^{-1} of cool-white fluorescent light. It is not necessary for the recovery conditions to be the same as the exposure conditions. However, the conditions listed are optimal and will result in the fastest cystocarp development.

Plants are not aerated during the exposure period. Chambers are either shaken at 100 rpm on a rotary shaker or briefly hand-swirled twice a day. Spermatia are not motile, therefore some water motion is critical. Aeration will inhance the growth rate of plants in the recovery bottles, although, adequate growth will occur using a shaker.

Nutrients

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

The nutrients recomended for natural seawater are not sufficient for healthy plants in artificial seawater. The nutrients for the GP2 artificial seawater are listed in Table 4. EDTA has not been elimi-

nated, but has been reduced to 20 ug/L (<1% of the original recipe). One of the reasons natural seawater is better than artificial seawater is probably due to the variety of natural organic chelators in the former. Therefore, elimination of all organic chelators from artificial seawater should not be necessary. In fact, total elimination of EDTA from artificial seawater can result in a greater sensitivity to toxicants such as copper when compared to results with natural seawater.

Effluent Concentrations

For end-of-the-pipe samples, the concentrations that are currently being used for Champia are 0.63, 1.25, 2.5, 5 and 10 % effluent plus a control. The concentrations recommended in the effluent handling addendum of this Guidance Manual are also acceptable.

Table 4. Recipe of nutrients to be added to GP2 artificial seawater for stock cultures and test medium. See footnotes for differences between test medium and culture medium. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 min (the pH is adjusted to 2.0 with HCl prior to autoclaving to prevent precipitation).

COMPOUND	AMOUNT/LITER STOCK CULTURES	AMOUNT/LITER CONCENTRATED STOCK[a]
$NaNO_3$	127 mg	12.7 g
$NaH_2PO_4 \cdot H_2O$	12.8 mg	1.28 g
$Na_2EDTA \cdot 2H_2O$	2.66 mg[b]	266 mg
$NaC_6H_5O_7$ (citrate)	1.03 mg	103 mg
Iron[c]	195 ug	19.5 mg
Trace Elements[d]		
$Na_2MoO_4 \cdot 2H_2O$	24.2 ug	2.42 mg
KI	83 ug	8.3 mg
$ZnSO_4 \cdot 7H_2O$	21.8 ug	2.18 mg
$NaVO_3$	6.1 ug	0.61 mg
$MnSO_4 \cdot H_2O$	0.61 ug	61.0 ug
Vitamins		
Thiamine·HCl	1.95 mg	
Biotin	1.0 ug	10 mL[e]
B_{12}	1.0 ug	

[a]Use 1.25 mL/L for test medium (with the adjusted EDTA concentration) and 5 mL/L for stock cultures. For test media an additional 2.5 mL/L of the sodium bicarbonate stock solution is added (see Table 3).

[b]The concentration is 20 ug/L for final solution of test media in artificial seawater; EDTA is omitted entirely if the test medium is natural seawater.

[c]The same stock solution as for Table 2.

[d]Trace elements are omitted for toxicity test medium (they are also generally omitted when this medium is used with natural seawater).

[e]Vitamin stock solution autoclaved separately in 10 mL sub-samples. Each 10 mL contains 195 mg of thiamine·HCl, 100 ug biotin, and 100 ug B_{12}. Adjust pH to ca 4.0 before autoclaving for 2 min.

PROTOCOL

1. Set up and label control and treatment chambers; three per treatment and controls.
2. Fill chambers with 100 mL of control or treatment water (28 to 30 °/₀₀). Alternately, all chambers can be filled with control water and the toxicant added with micropipets. For toxicant volumes exceeding 1 mL, adjust amount of dilution water to give a final volume of 100 mL.
3. Add the appropriate nutrients and bicarbonate to each chamber (see Table 2 or 4).
4. Add five female branch tips and one male branch to each chamber. Make sure the toxicant is present before the male is added.
5. Place chambers under cool-white light (ca 75 uE m^{-2} s^{-1}) at 22 to 24 °C. Place a thermometer in a flask of water among the chambers.
6. Gently hand-swirl chambers twice a day. Alternately, shake continuously at 100 rpm on a rotary shaker. Record temperature daily.
7. If desired, media can be changed after one day (24 hr).
8. After 2 days (48 hr):
   A. Label recovery bottles (these can be almost any type of container or flasks with 200 to 400 mL of natural seawater plus the additional nutrients (see Table 2 or 4). Smaller volumes can be used, but should be checked to make sure that adequate growth will occur without having to change the medium. As with culture vessels, all glassware should be acid-stripped with 10 to 15 % HCl.
   B. With forceps, gently remove females from test chambers and place into recovery bottles. Add aeration tubes and foam stoppers.

C. Place bottles under cool-white light (at the same irradiance as the stock cultures) and aerate for the 5- to 7-day recovery period. If recovery is on a shaker, then eliminate aeration tubes and reduce the volume of seawater to approximately one-half of the vessel volume (this will enhance the water motion).

9. At the end of the recovery period count the number of cystocarps per female and record the data (Addendum C-V). Cystocarps are counted by placing females between the inverted halves of a polystyrene petri dish with a small amount of seawater (to hold the entire plant in one focal plane). Using a stereo-microscope, the emergent cystocarps can be easily counted. Cystocarps are distinguished from young branches because they possess an apical ostiole (opening for spore release) and darkly pigmented spores (see Figs. 7,8). One of the advantages to this test procedure is that if there is uncertainty about the identification of an immature cystocarp, then the plants can just be aerated for a little longer in the recovery bottles. Within 24 to 48 hr the structure in question will either look more like a mature cystocarp; look more like a young branch; or have changed very little, if at all (i.e., it is an aborted cystocarp). No new cystocarps will form since the males have been removed, the plants will only get bigger. Occasionally, cystocarps will abort, and these should not be included in the cystocarp counts. Aborted cystocarps are easily identified by their dark pigmentation (Fig. 9). They also often begin to form a new branch at their apex.

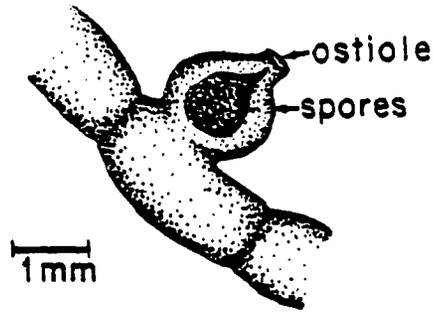

Figure 7. A mature cystocarp of Champia parvula. In the controls and the lower effluent concentrations, cystocarps are often clustered in groups of as many as 10 to 12.

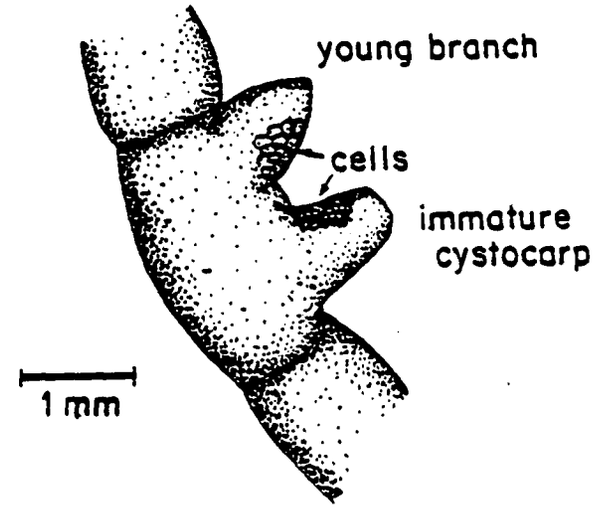

Figure 8. Comparison of a very young branch with an immature cystocarp. Both can have sterile hairs and the young branch may or may not have trichogynes. However, the immature cystocarps will never have trichogynes. Young branches are usually more pointed at the apex and will not form an ostiole. The cell dimensions of young branches are larger than those of the cystocarp.

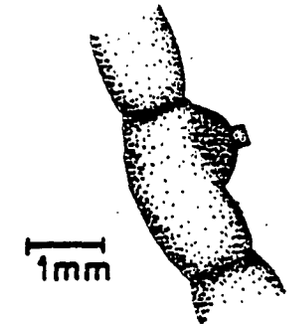

Figure 9. An aborted cystocarp of Champia parvula. A new branch will eventually develop at the apex.

STATISTICAL TREATMENT OF DATA

Chronic values are calculated using two different criteria for determining significant differences. One criterion for difference from controls is absence of cystocarps; $\geq$ 95% decrease from the control (Thursby and Steele, 1986). The other criterion is based on statistical differences. Statistically significant decreases in the number of cystocarps are determined by one-way analysis of variance (ANOVA) followed by Dunnett's mean separation test (alpha = 0.05) for comparison of treatments with a control (Steel and Torrey, 1960). The results from each replicate chamber are reported as the mean number of cystocarps per plant (n=3 for each treatment).

Chronic values are expressed either as the no-effect range, the lowest concentration that results in a significant difference from the control and the next lowest concentration tested, or as the geometric mean of these two values (Buikema, et al., 1982). If the lowest concentration that results in a significant difference is the lowest concentration tested, then the geometric mean is not calculated. One can only report the lowest concentration that gave the significant difference (one should also consider repeating this test). In practice, the "absence of cystocarps" endpoint is generally used for determining the effect concentration from an effluent diluton series. However, if the concentration that causes a statistical decrease from the control is also reported, then some idea of the steepness of the dose response curve can be inferred. The statistical difference is used primarily when testing receiving waters where dilutions are usually not made.

CRITERIA FOR ACCEPTABILITY

1. A test is not acceptable if control mortality exceeds 20% (generally there is no control mortality).
2. If plants fragment in either the controls or the lowest exposure concentration so that individual plants can not be identified, then the test is not acceptable. This is not critical if absence of sex is the only endpoint of interest. However, the fact that the plants fragmented indicates they are not at their best and the data may be biased toward the lower concentrations.
3. A test should not be considered definitive if the controls average fewer than 10 cystocarps per plant. If no sexual reproduction occurs in the controls, then this test can not be considered acceptable.
4. If the plants in the two control chambers are suspected of responding differently (this can be checked with a t-test), then the test should not be considered acceptable.
5. The data from all replicates at the effluent concentration determined to be the effect concentration should be statistically equal. That is, all replicates should show the effect.

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ADDENDUM C-I

List of materials for performing toxicity tests with Champia parvula for effluent program.

1. Fine-point stainless steel forceps.
2. 100 mL polypropylene cups with covers (or 125 mL Erlenmeyer flasks) to be used as exposure chamabers.
3. Polystrene petri dishes to hold plants for counting cystocarps and for cutting branch tips.
4. 100 mL graduated cylinder to measure control and treatment volumes.
5. 1 and 10 mL disposable pipets.
6. Digital micropipets (200 and 1000 uL maxima) if dilutions are made directly in test chambers.
7. Disposable tips for micropipets.
8. Nutrient and sodium bicarbonate stock solutions.
9. Recovery bottles or flasks, need one per treatment or control chamber.
10. Aquarium pump(s) and air tubing.
11. Plastic aeration tubes (1 mL disposable pipets work fine) and foam plugs.
12. Thermometer and flask or bottle to hold it.
13. Marking pens and colored tape.
14. Cool-white fluorescent lighting, sufficient to give 75 uE m^{-2} s^{-1} (ca 500 foot-candles).
15. Rotary shaker for exposure chambers (hand-swirling twice a day can be substituted).
16. Stereomicroscope for counting cystocarps.
17. Refractometer for salinity measurements.
18. Data sheets (one per test).
19. Protocol.

ADDENDUM C-II

Summary of test conditions for Champia parvula sexual reproduction test.

1. Test type:	Static, non-renewal
2. Salinity:	30°/$_{oo}$
3. Temperature:	22 to 24 °C
4. Photoperiod:	16h light:8h dark
5. Light source:	cool-white fluorescent
6. Irradiance:	ca 75 uE m^{-2} s^{-1}
7. Test solution volume:	100 mL
8. Test chamber size:	110 mL polypropylene cups (with covers) or 125 mL Erlenmeyer flasks
9. Number of test organisms per test chamber:	5 female branch tips and one male
10. Number of replicate chambers per treatment:	3
11. Aeration:	None; chambers are either shaken at 100 rpm on a rotary shaker or hand-swirled twice a day
12. Dilution water:	30 °/$_{oo}$ natural or artificial seawater with additional nutrients added
13. Test duration:	2 day exposure followed by a 5- to 7-day recovery period for cystocarp development
14. Effect measured:	Sexual reproduction (number of cystocarps per female)

ADDENDUM C-III

Equations for making salinity adjustments.

A. To dilute to a desired salinity with deionized water.

$$S_i \times V_i = S_f \times V_f$$

where:

S_i = initial salinity (measured)

S_f = final salinity (selected)

V_i = initial volume (unknown)

V_f = final volume (selected)

Solve for V_i, then dilute to the final (selected) volume with deionized water.

B. To mix two different salinities to get a third salinity.

$$S_I(V_f - V_{II}) + S_{II} \times V_{II} = S_f \times V_f$$

and

$$V_f = V_I + V_{II}$$

where:

S_I = salinity of the first solution (measured)

S_{II} = salinity of the second solution (measured)

S_f = salinity of the final solution (selected)

V_I = volume of the first solution (unknown)

V_{II} = volume of the second solution (unknown)

V_f = volume of the final solution (selected)

Solve the first equation for V_{II}, then solve the second equation for V_I.

Example: Solution I salinity = 10 °/₀₀;
solution II salinity = 90 °/₀₀;
final solution salinity desired = 30 °/₀₀;
final volume wanted = 1000 mL.

First equation becomes: $10\ (1000 - V_{II}) + 90\ V_{II} = 30 \times 1000$

$$10{,}000 - 10\ V_{II} + 90\ V_{II} = 30{,}000$$

$$80\ V_{II} = 20{,}000$$

$$V_{II} = 250\ mL$$

Second equation becomes:

$$V_{I} = 1000 - 250$$

$$V_{I} = 750\ mL$$

Any of the three volumes can be selected as the constant, solving for the other two volumes. For example you may have 500 mL of solution II and wish to know how much of solution I to add to get 30 °/₀₀. In this case you would just solve the first equation for V_f and continue from there.

ADDENDUM C-IV

Precision testing for Champia parvula short-term.

Intralaboratory precision testing of the short-term exposure reproductive test with Champia parvula has not been conducted yet. However, we have several repeat tests with effluents and one single compound (copper). All tests concentrations were unmeasured. The concentration given is the geometric mean of the effect/no-effect level. Effect is defined as having any number of cystocarps/plant $\leq$ 5% of the control. In general, the agreement among the repeat tests was very good. For the heavy metal effluent, the variation was by a factor of 3, but the two tests (on the same sample) were run approximately 2 months apart.

COMPOUND	COLLECTION DATE	TEST DATE	EFFECT LEVEL
Copper	--	2/18/85	8.8 ug/L
	--	2/18/85	8.8
	--	4/3/85	8.8
	--	4/3/85	8.8
	--	6/7/85	8.8
Heavy Metal Effluent	8/17/84	9/25/84	1.76 %
	8/17/84	11/27/84	0.56
Organic Effluent			
	5/8/85	5/16-shaken	14.1 %
	5/8/85	5/16-not shaken	14.1

ADDENDUM C-V

CHAMPIA PARVULA

CYSTOCARP DATA SHEET

COLLECTION DATE _____ RECOVERY BEGAN (date) _____

EXPOSURE BEGAN (date) _____ COUNTED (date) _____

EFFLUENT OR TOXICANT _____

TREATMENTS (% EFFLUENT, µG/L, or REC. WATER SITES)

REPLICATES	CONTROL						
A 1							
2							
3							
4							
5							
MEAN							
B 1							
2							
3							
4							
5							
MEAN							
C 1							
2							
3							
4							
5							
MEAN							
OVERALL MEAN							

Temperature _____

Salinity _____

Light _____

Source of Dilution Water _____

RECEIVING WATER SUMMARY SHEET

SITE _____

COLLECTION DATE _____

TEST DATE _____

LOCATION	INITIAL SALINITY	FINAL SALINITY	SOURCE OF SALTS FOR SALINITY ADJUSTMENT *

* i.e. natural seawater brine, GP2 brine, GP2 salts, etc.
(include some indication of amount)

COMMENTS:

PART II

COMPARISON OF SHORT- AND LONG-TERM SEXUAL REPRODUCTION TESTS WITH THE MARINE RED ALGA *CHAMPIA PARVULA*

Abstract--A two-day exposure test using the marine red alga Champia parvula has been developed for assessing the toxic effects of complex effluents entering the marine and estuarine environments. The initial exposure was followed by a 5- to 7-day recovery period to allow the development of any cystocarps (evidence of sexual reproduction--the endpoint measured). The two-day exposure test was validated by comparison with a previously developed two-week test in which "no sexual reproduction" (NSR) was used as the reproductive endpoint measured. Single compounds can be more accurately tested with the two-week test procedure than effluents, therefore, they were used to compare the two-day exposure test with the two-week exposure. A total of nine single compounds were tested using the two testing procedures. Concentrations that resulted in a 95% or greater decrease from the control response with the two-day exposure were considered the best estimate of the NSR response from the two-week test. All of the single compounds tested, except cadmium, yielded essentially the same results with both tests.

Keywords--Champia, effluents, sexual reproduction

INTRODUCTION

The Permits Division of the U.S. Environmental Protection Agency's (EPA) Office of Water needs toxicity test methods for marine and estuarine species. The U.S. EPA's Environmental Research Laboratory at Narragansett, Rhode Island has developed or modified four toxicity tests to begin to address the above need. The four species used are: the red macroalga, Champia parvula; the sea urchin, Arbacia punctulata; the mysid, Mysidopsis bahia; and the sheepshead minnow, Cyprinodon variegatus. The methods are needed to characterize the toxicity of effluent discharges within the National Pollutant Discharge Elimination System (NPDES). Since March 9, 1984, EPA has had the authority to require biological testing as a condition for issuing NPDES permits [1]. These tests should yield chronic data in a relatively short time period (7 days or less). It is also necessary that the methods be simple and cost efficient (requiring standard hardware and laboratory facilities). The species used should be readily available, and should also be practical for both on-site and off-site testing. All of the requirements are met by the test method for the marine red macroalga, Champia parvula.

Seaweeds have been considered less useful for toxicity testing than microalgae [2], and microalgae are often considered less sensitive than aquatic animals [3,4]. Therefore, one could easily come to the erroneous conclusion that toxicity testing with seaweeds is not necessary. Recently the statement was made that "seaweeds seem to be rather insensitive to many chemicals and will probably survive pollution better that many other organisms in the marine environment" [2].

Previous conclusions about seaweed sensitivity were based on only a few species, and generally considered only vegetative growth of the

macroscopic life history stage as the endpoint. The sensitivity of seaweeds can increase when sexual reproduction is used as an endpoint, and can be greater than many aquatic animals that have been tested. This has been shown for the red alga, Champia parvula [5-7], as well as with the brown algae Fucus edentatus and Laminaria saccharina [8]. In addition, using growth of young sporophytes, L. saccharina has been placed among the most sensitive marine organisms for toxicity of copper, zinc and mercury [9].

A two-week toxicity test has already been developed for the macroalga, Champia parvula, to assess chronic effects of pollutants to marine seaweeds [5]. The test has been evaluated with heavy metals [5], arsenite and arsenate [6] and ten different organic compounds [7]. Sexual reproduction was generally the most sensitive and practical endpoint to use for C. parvula. The two-week exposure, however, made this test procedure unacceptable for testing complex effluents and their receiving waters.

Effluents can not be easily tested using the two-week test procedure. The two-week toxicty test with Champia parvula requires that the cultures remain unialgal during the test period. Fine-filtering or autoclaving, which would be necessary to eliminate unwanted microalgae, could change the character of the effluent or receiving water. Any microalgae introduced with the effluent would compete with C. parvula for light and nutrients, thus influencing C. parvula's growth rate. However, if sexual reproduction is used as the sole endpoint, then plants only need to be exposed for a few days (long enough to show effects on fertilization). Any effect of other organisms on the growth rate of C. parvula should not be serious since interest would only be in whether sexual reproduction had taken place. The two-week procedure

also requires that the media be changed during the test. Therefore, either the effluent sample would have to be stored (i.e. refrigerated) or additional samples would have to be collected during the test period. Both of these alternatives would result in variable toxicity. Single compounds can be more accurately tested with the two-week test procedure than effluents, therefore, they were used to compare the two-day exposure test with the two-week exposure.

This paper describes a method which uses sexual reproduction to estimate chronic toxicity after a short-term exposure to toxicants. In brief, the method consists of exposing males and females to effluents or receiving waters for two days, followed by a 5- to 7-day recovery period in control medium. The recovery period allows time for any cystocarps to mature. At the end of the recovery period the number of cystocarps per plant are counted.

MATERIALS AND METHODS

Maintenance of stock cultures

Unialgal stock cultures of both male and female gametophytes of Champia parvula (C. Agardh) Harvey were maintained in separate, aerated 1000 mL Erlenmeyer flasks containing 800 mL of culture medium. All culture glassware was acid-stripped in 10 to 15 % HCl and rinsed in deionized water after washing. The culture medium was made from natural seawater (from lower Narragansett Bay, RI) to which additional nutrients were added (Table 1). The seawater was filtered through a 15-μm charcoal filter and a 0.3-μm Balston filter, then autoclaved for 30 min at 15 psi in 20 L carboys. The culture flasks were capped with aluminum foil and autoclaved dry for 10 min. Culture medium was formulated by dispensing seawater into the sterile flasks and adding the appropriate nutrients from a sterile stock solution.

Initial stock cultures were started weekly with about twenty 0.5 to 1.0 cm branch tips. Cultures were gently aerated through sterile, cotton-plugged, disposable, polystyrene 1 mL pipets. Cultures were capped with foam plugs and aluminum foil and illuminated from the side with 75 $\mu E\ m^{-2}\ s^{-1}$ of cool-white fluorescent light on a 16h:8h, light:dark cycle. The temperature was 22 to 24 °C and the salinity 28 to 30 $^{\circ}/_{\circ\circ}$. Media were changed once each week.

About half of the plants were discarded (or placed into another culture vessel) with each weekly medium change to reduce the amount of biomass as the plants grew. At the end of three weeks plants were ready to use for testing. Readiness was defined as having enough plant material to perform at least one test. With this procedure, actively growing plants were continuously available.

A stock culture was not used as a source of test material if the plants appeared to be stressed or undernourished. Under conditions of stress the tips of the branches turned "pink" and the older tissue was generally much paler. Under conditions of nutrient deficiency (resulting usually from too much plant material in the culture flask or too long since the last medium change) the entire plant turned pale yellow. If the stress was severe enough the older tissues (main axes) or occasionally the branch tips turned white (evidence of necrotic tissue).

Two-week test

All procedures for the two-week test followed those previously described [5], except EDTA was omitted from the medium and vitamins were added. The test medium was then identical to that used for the two-day exposure test. Toxicity test duration was 14 days. Tests were performed with 400 mL volumes in 500 mL, screw-capped Erlenmeyer flasks. The medium was ½ strength of that used for the stock cultures. In addition 150 mg/L sodium bicarbonate was added rather than aeration. Flasks were shaken on a rotary shaker at 100 rpm. Media were replaced on days 7, and 11. All other conditions were the same as those for stock cultures.

After stock cultures were rinsed in sterile seawater to remove traces of old medium, 2- to 3-mm branch tips were cut from females to serve as inocula for toxicity tests. Five branch tips were placed into each test flask. One male branch (about 1 cm long), visibly producing spermatia, was added to each flask containing females. Replicate flasks were used for each treatment. At the termination of each toxicity test, females were examined for the presence of cystocarps (evidence of sexual

reproduction). The concentration which resulted in no sexual reproduction (NSR) was determined.

Two-day exposure

Stock cultures were rinsed in sterile seawater to remove traces of old medium. Female branch tips, 7 to 10 mm in length, were cut to serve as inocula. Five tips were placed into each treatment cup, along with one male branch (1.5 to 2.0 cm long), visibly producing spermatia. Tests were performed in replicate 100 mL polystyrene cups, with plastic caps, containing 80 mL of medium. The nutrient medium was the same as for the two-week tests.

The two day exposure was followed by a 5- to 7- day recovery period (for females only) in control medium for cystocarp development. Temperature, salinity and light conditions were the same as for stock cultures, except that light was from above. Plants were not aerated during the exposure period. Exposure chambers were shaken at 100 rpm on a rotary shaker. Recovery bottles were aerated, since this enhanced the growth rate of plants and therefore the rate of development of cystocarps.

At the end of the recovery period the number of cystocarps per female were counted. The results from each replicate cup were reported as the mean number of cystocarps per plant (n = 2 for the treatment). The data were examined for NSR, 95 and 50% decreases from the control, and statistical differences from the control. Statistical decreases in the number of cystocarps were determined by one-way analysis of variance (ANOVA) followed by Dunnett's means separation test (alpha = 0.05) for comparisons of treatments with a control [10].

One advantage of this test procedure is if there is uncertainty

about the identification of an immature cystocarp, then the plants can just be aerated a little longer in the recovery bottles. No new cystocarps will form since the male gametophytes have been removed; the plants will only get bigger.

Toxicant concentrations

The highest concentration tested was based on preliminary experiments and was chosen to cause death or a near death response after a two-week exposure. The dilution factor for all test runs was 0.6. Only one stock solution was prepared for each toxicant. All concentrations were obtained by dispensing from these stock solutions with adjustable micropipets.

Stock solutions of sodium arsenite, copper sulfate, cadmium chloride and silver nitrate were prepared in deionized water. Toxaphene was dissolved first in acetone and then diluted with triethylene glycol (TEG) to give a final acetone to TEG ratio of 1 to 20. Pentachloroethane and pentachlorophenol were prepared in TEG alone. Benzene and isophorone were dispensed directly. For benzene, isophorone and pentachloroethane (all liquids), density was used to calculate the weight of the compound in solution. A carrier control (at the highest concentration of carrier used with a toxicant) was used with those toxicants that required a carrier for solubility. Water samples from the test chambers were not chemically analyzed for toxicant concentrations, therefore all concentrations reported are nominal concentrations added.

RESULTS AND DISCUSSION

The results of the comparison of the two-day exposure test with the two-week test using single compounds are shown in Table 2. Only data for concentrations causing no sexual reproduction (NSR) are reported for the two-week test. Several values are listed for the two-day exposure test. The object of the two-day exposure was to estimate the chronic effect from the two-week test. The endpoint of choice for the two-week test was NSR, therefore all two-day results are compared only to this number. The same concentrations were tested in both test procedures, and for four of the nine compounds the NSR endpoint was not achieved for the two-day test.

The best two-day values for estimating the two-week NSR results were those from the concentration that resulted in a 95% or greater decrease in the number of cystocarps (0 to 5% of control) when compared to the controls. This is seen more clearly in Table 3 which shows the ratios of the values in Table 2 to the two-week values. Cadmium was the only compound tested that did not give a good relationship between the two-day and the two-week results. Cadmium is generally a slow acting toxicant [11, 12] and two days may not have been enough time to elicit its effect. The ratios for arsenite and silver are close to one, while the remaining are one. Arsenite and silver were the only two compounds for which the two test procedures were not started on the same date. It should be noted that the two-day benzene test was performed in 400 mL, screw-capped Erlenmeyer flasks instead of the polystyrene cups. When plants were exposed in 80 mL test solution the toxicity was much less, probably due to the high volatility of benzene. If volatile compounds are being tested, then larger, air-tight exposure chambers should probably be used. However, the two day test procedure is designed for

testing effluents and receiving waters. Highly volatile compounds are significantly reduced in pretreatment before effluents are discharged. Therefore the failure of the test with benzene when using smaller, non-air-tight vessels is not expected to be a problem when testing effluents.

A sample data set is graphed for pentachloroethane in Figure 1, illustrating that a 95% decrease from the control is a good value for estimating the two-week results. The dose-response curves for both the two-week test and the short-term exposure tests are similar. However, no cystocarps were produced during the two-week test at 13,000 or 22,000 $\mu g\ L^{-1}$, whereas a few were produced at both concentrations during the short-term test. A similar relationship between the two-day an the two-week results was seen for most of the compounds tested.

Sexual reproduction was selected as the endpoint for two-day testing for several reasons. It was proven previously to be a sensitive and practical endpoint from the two-week toxicity test procedure [5-7]. A sexual reproduction test for toxicity could be short enough to fit the time constraints for tests used in the effluent program (ca. 7 days). Finally, Champia parvula is an annual plant and inhibition or absence of sexual reproduction reduces or eliminates the next stage in the life history. Total absence of cystocarp formation is the easiest endpoint to interpret as far as field populations are concerned. In most of the red algae, each fertilization results in the formation of a new life history stage, the carposporophyte, "parasitic" on the female and housed within the cystocarp. Each carposporophyte is capable of producing many spores (perhaps a hundred or more in the case of C. parvula). This characteristic makes it difficult to interpret the biological signif-

icance of a statistical decrease in the number of cystocarps or an arbitrary percent decrease such as 50%. Absence of reproduction leaves no doubt about its biological significance.

The two-day exposure test has been used successfully in both a mobile laboratory and in the main laboratory (EPA, Environmental Research Laboratory, Narragansett, RI, USA). The method is easy and cost-efficient to perform. Stock cultures are maintained in the laboratory with standard laboratory equipment, therefore, plant material can be available throughout the year. The test procedure is intended to be used to estimate chronic effects of complex effluents on marine macroalgae, although it can obviously be used for single compounds. The procedure has already been included in a draft guidance manual for testing marine and estuarine effluents. Comparing this test with a two-week chronic test using single compounds has shown that the test can be used for determining adverse effects on sexual reproduction in the marine alga, Champia parvula.

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Table 1. Recipe for additional nutrients to be added to natural seawater for Champia parvula stock cultures and test medium. Both EDTA and trace metals have been omitted. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 minutes.

COMPOUND	AMOUNT/LITER	
	TEST MEDIUM CONC.	CONCENTRATED STOCK[a]
$NaNO_3$	9.35 mg	3.74 grams
$NaH_2PO_4 \cdot H_2O$	0.62 mg	0.25 grams
Iron[b]	2.6 ug	1.04 mg
Vitamins		
B12	0.06 ug	
Biotin	0.06 ug	10 mL[c]
Thiamine•HCl	12.5 ug	

[a]Use 0.2 mL/80mL (2.5 mL/L) for test medium concentrations and 10 mL/L for stock cultures. For test medium only, add 0.2 mL/80mL of a sodium bicarbonate solution. A stock solution of 60 mg/mL sodium bicarbonate is prepared by autoclaving it as a dry powder and then dissolving it in sterile deionized water.

[b]Iron stock solution prepared by dissolving 1 g iron powder in 10 mL concentrated HCl plus ca. 1 mL of deionized water. This is diluted to 1 liter with deionized water. Acceptable stock solutions can also be made with ferric or ferrous chloride.

[c]Vitamin stock solution autoclaved (2 min) separately in 10 mL subsamples. Each 10 mL contains 24 µg B12, 24 µg biotin and 5 mg thiamine•HCl. Adjust pH to ca. 4.0 before autocalving.

Table 2. Test results for Champia parvula comparing the two-day exposure test and the two-week test using single compounds. Values listed are the geometric means of the effect and the no-effect concentrations in ug/L. NSR refers to no sexual reproduction, i.e. no cystocarps were formed at these concentrations. The other column headings refer to concentrations that resulted in 95 and 50% decreases from controls, and concentrations that resulted in a number of cystocarps statistically less than controls.

COMPOUND	TWO-DAY EXPOSURE				TWO-WEEK
	NSR	95%	50%	Stat. Dif.	NSR
Arsenite[a]	>300	230	84	84	140
Copper	7.7	4.6	1.0	<0.8	4.6
Cadmium	>100	>100	>100	17	77
Silver[a]	1.5	0.9	0.9	0.5	1.5
Benzene[b]	73,600	73,600	<34,300	44,250	73,600
Pentachloroethane	>21,800	10,200	2,200	<1,700	10,200
Pentachlorophenol	465	465	280	280	465
Toxaphene	140	140	84	84	140
Isophorone	>138,500	107,300	38,300	38,300	107,300

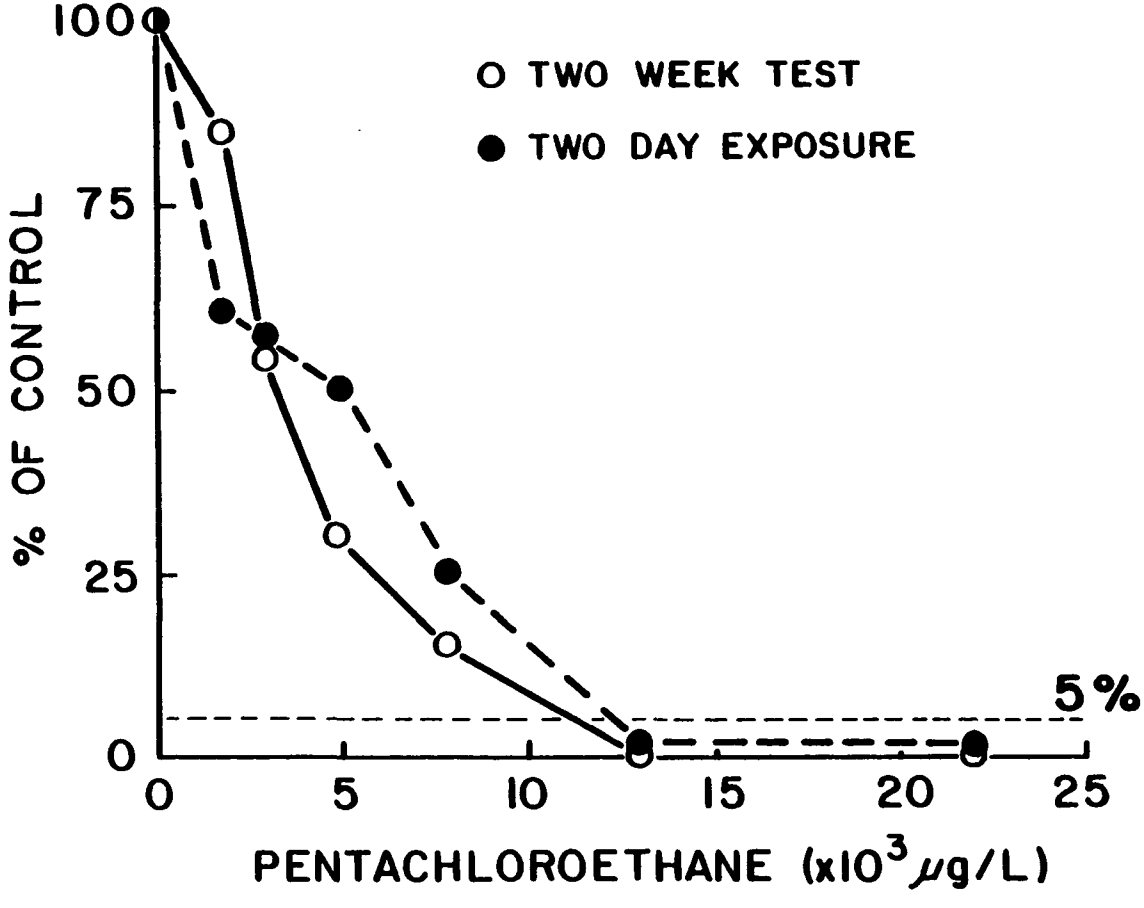
[a]Short-term exposure and two-week test not run silmultaneously.

[b]Both short-term exposure and two-week test run in 400 mL volume.

Table 3. Ratio of two-day exposure results and two-week test results from Table 2 for Champia parvula. See Table 2 for explanation of column headings.

COMPOUND	NSR	5%	50%	Stat. Dif.
Arsenite	>2.1	1.6	0.6	0.6
Copper	1.8	1.0	0.2	<0.2
Cadmium	>1.3	>1.3	>1.3	0.2
Silver	1.0	0.6	0.6	0.3
Benzene	1.0	1.0	<0.5	0.6
Pentachloroethane	>2.1	1.0	0.2	<0.2
Pentachlorophenol	1.0	1.0	0.6	0.6
Toxaphene	1.0	1.0	0.6	0.6
Isophorone	>1.3	1.0	0.4	0.4

Figure 1. Comparison of the two-day exposure test results and the two-week test results for Champia parvula using pentachloroethane. The graph illustrates the use of the 95% decrease as the cut off point for the two-day exposure test. Note that some cystocarps were produced at the two highest concentrations during the two-day exposure, but not during the two-week test.


100
75
50
25
0
% OF CONTROL
O TWO WEEK TEST
● TWO DAY EXPOSURE
5%
0
5
10
15
20
25
PENTACHLOROETHANE (x10³ µg/L)

PART III

SUMMARY OF RESULTS FROM TESTING COMPLEX EFFLUENTS

INTRODUCTION

Tests have been conducted on-site in a mobile laboratory and at the EPA's Environmental Research Laboratory, Narragansett, RI. Several different types of complex effluents have been tested over the past two years. These include one from a pulp mill; two industrial sites that discharge effluents containing heavy metals; five industrial sites discharging organically contaminated effluents, including pestcides and dyes; and 17 different sewage effluents. In all, over 100 tests were conducted.

More complete data is included for the pulp mill effluent (Tables 2 and 3, and Figures 1 and 2) from ITT Rayonier in Fernadina Beach, Florida. These results may be incorporated into the first marine toxicity-based NPDES permit.

MATERIALS AND METHODS

The test procedure used is described in the guidance manual (Part I of this report). In brief, the method consists of exposing males and females to effluents or receiving waters for two days, followed by a five- to seven-day recovery period in control medium. The recovery period allows time for any cystocarps to mature. At the end of the recovery period the number of cystocarps per plant are counted. Some of the earliest tests differed in that the exposure period was four days instead of the now standard two days. However, our earlier work also showed that two and four day exposures yielded essentially the same results.

Chronic values can be calculated using two different criteria for determining significant differences. One criterion for difference from controls is absence of cystocarps; $\geq$ 95% decrease from the control. The

other criterion is based on statistical differences. See Parts I and II of this report for a further explanation.

RESULTS

Table 1 summarizes all of the effluent test results to date. The summary is based on absence of reproduction as the measurement of the effect. In general the effects could be separated based on effluent type. Heavy metal containing effluents were by far the most toxic, followed by oranic and pulp mill effluents, and then sewage effluents.

Tests on the pulp mill effluent from ITT Rayonier were performed on four separate effluent and receiving water collections (May 15, 17, 18 and 19, 1986). The tests set up on May 15th and 19th are not included in these results. The control values from these two runs were unacceptable (an average of <10 cystocarps per plant). The May 15th run used plants cultured in artificial seawater, these plants are more difficult to judge as ready-to-use in the absence of a compound microscope. The test runs on the other three days all used plants cultured in natural seawater (where color of the tissue is a good indicator of "readiness"). However, the test run set up on the 19th lasted only one day instead of the usual two.

Tables 3 and 4 list the data from the May 17th and 18th collections for the effluent and receiving waters respectively. Figure 1 is a graph of the May 18th data against percent effluent, based on the previous week's dye study. These results indicated that the effect concentrations in the receiving water was consistent with that determined in the effluent test. Figure 2 is a graph of the May 17th and 18th effluent data and data from an ammonium chloride toxicity test run started on June 11, 1986. The similarity between the two curves

suggests that ammonia may be the main toxicant in the ITT Rayonier effluent that inhibits sexual reproduction in Champia. This conclusion supports the chemical fractionation studies conducted on-site by EPA's Duluth laboratory inwhich unionized ammonia was the primary toxic comnponent of the effluent.

Table 1. Summary of the results for all the effluents tested. The values represent the range of response for the No Sexual Reproduction effect ($\geq$ 95% decrease from the control). The number in parenthesis after the effluent type is the number of effluents of that type tested.

EFFLUENT TYPE	RANGE OF RESPONSE (%)
Heavy Metals (2)	0.05-0.5
Pulp Mill (1)	1.8
Organics (5) (pesticides, dyes, etc.)	3-20 (generally <5)
Sewage (17)	2.5-25 (generally 5-10)

Table 2. The effect of effluent from ITT Rayonier on formation of cystocarps by Champia parvula. Temperature was 23 to 25 °C, salinity was 30°/$_{oo}$, and light density was ca 100 uE m^{-2} s^{-1} of daylight fluorescent light on a 16h:8h, light:dark cycle. The concentration resulting in no sexual reproduction (≥95% decrease from control) was 2.5 % effluent with both test runs. The data from the May 15th and 19th test runs also had 2.5 % as the cut off point even though these data were considered unacceptable. No necrotic tissue was observed at any of the concentrations tested.

% Effluent	Number of Cystocarps per Plant (n=3)	
	May 17	May 18
Control	11 ± 2	14 ± 5
0.5	12 ± 3	13 ± 3
1.0	13 ± 1	9 ± 3
2.5	0.2	0
5.0	0	0
10.0	0	0

Table 3. The effect of receiving waters from the Amelia River on the formation of cystocarps by Champia parvula. Temperature, salinity and light conditions were the same as for the tests in Table 1. Effluent percents were calculated based on the dye study. No necrotic tissue was observed in any of the treatments. A blank space means missing data.

		Number of Cystocarps per Plant						
		May 17 Replicate[a]				May 18 Replicate		
Sta. No.	% Eff.	A	B	C	Mean	A	B	Mean
1	2.0	0.4	0.8	0.2	0.5*	0.0	1.6	0.8*
2	b	17.2	12.2	14.6	14.7	20.6	17.4	19.0
3		1.0	1.6	0.4	1.0*	2.0	4.0	3.0*
4	1.5					11.8	9.0	10.4
5		4.6	1.6	0.6	2.3*	0.0	1.2	0.6*
6	0.05					1.6	0.6	1.1*
7	1.4					2.4	4.4	3.4*
8	0.08	1.6	11.6	11.6	8.3	23.6	18.4	21.0
9	1.4					1.2	1.0	1.1*
10	0.5	14.4	15.4	15.8	15.2	10.4	16.6	13.5
11	0.2	10.8	13.2	14.4	12.8	26.6	13.3	19.9
12	0.9							
13	0.7					8.4	6.0	7.2
14	0.5					2.0	8.6	5.3*
15	0.5					5.8	4.6	5.2*
16						6.0	9.8	7.9
17	0.6					12.0	11.4	11.7
18	0.5					7.8	11.6	9.7
19	0.4					15.0	5.8	10.4
20	0.4					10.8	9.6	10.2
21	0.5					9.2	10.0	9.6

[a] Mean of five plants per replicate.

[b] Control station, assume effluent % to be close to zero.

* Statistically less than station 2 (ANOVA followed by Dunnett's mean separation test).

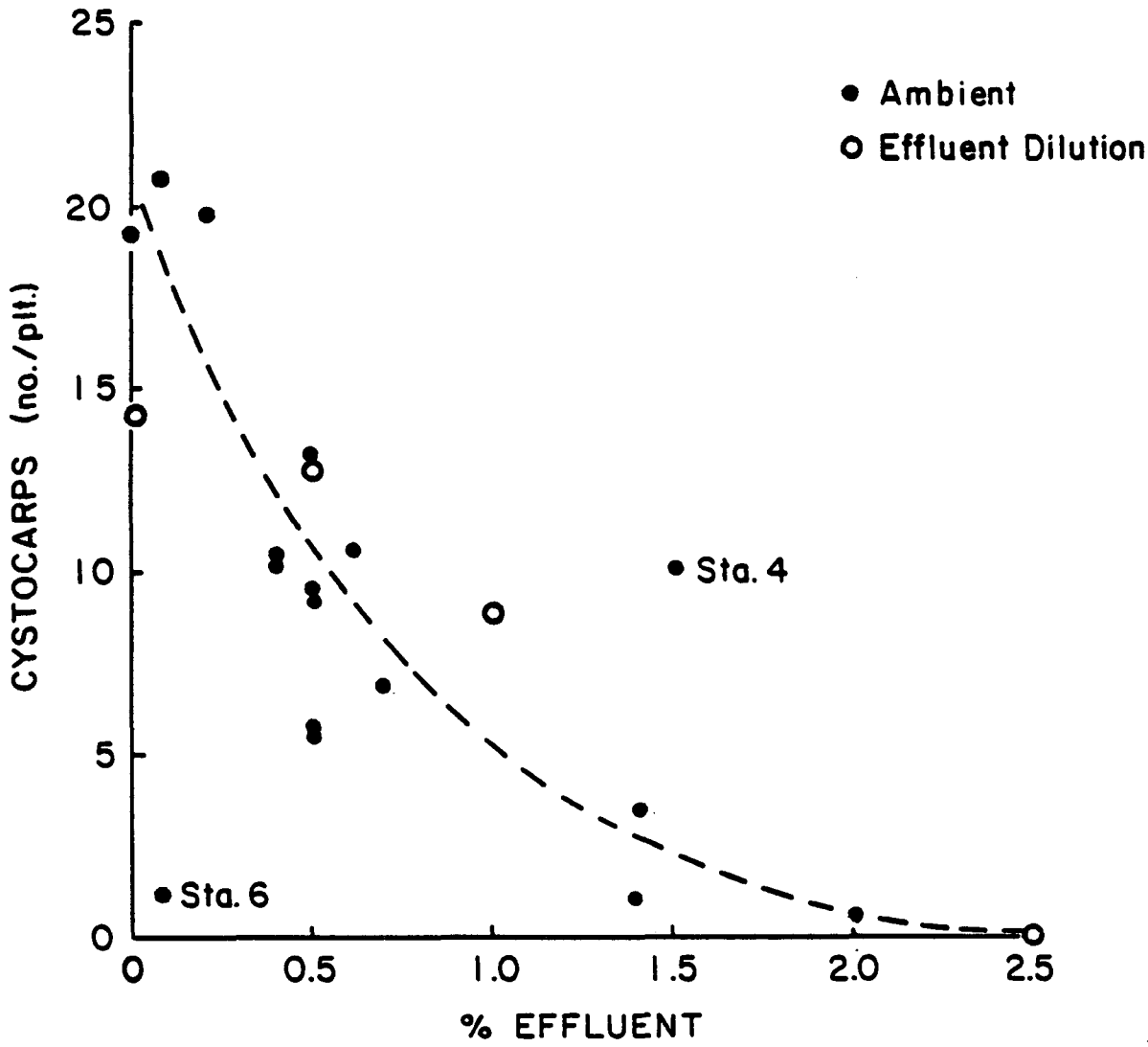


Figure 1. Number of cystocarps plotted againsts % effluent for data from May 18th. The % effluent was based on the dye study of the previous week. Plants treated with receiving water from station 4 had an unusually large number of cystocarps for the effluent %. Plants treated with water from station 6 had an unusually low number of cystocarps. This may be due to the time difference between the dye study and the toxicity testing. However, considering this time difference, the rest of the data show a good correlation.

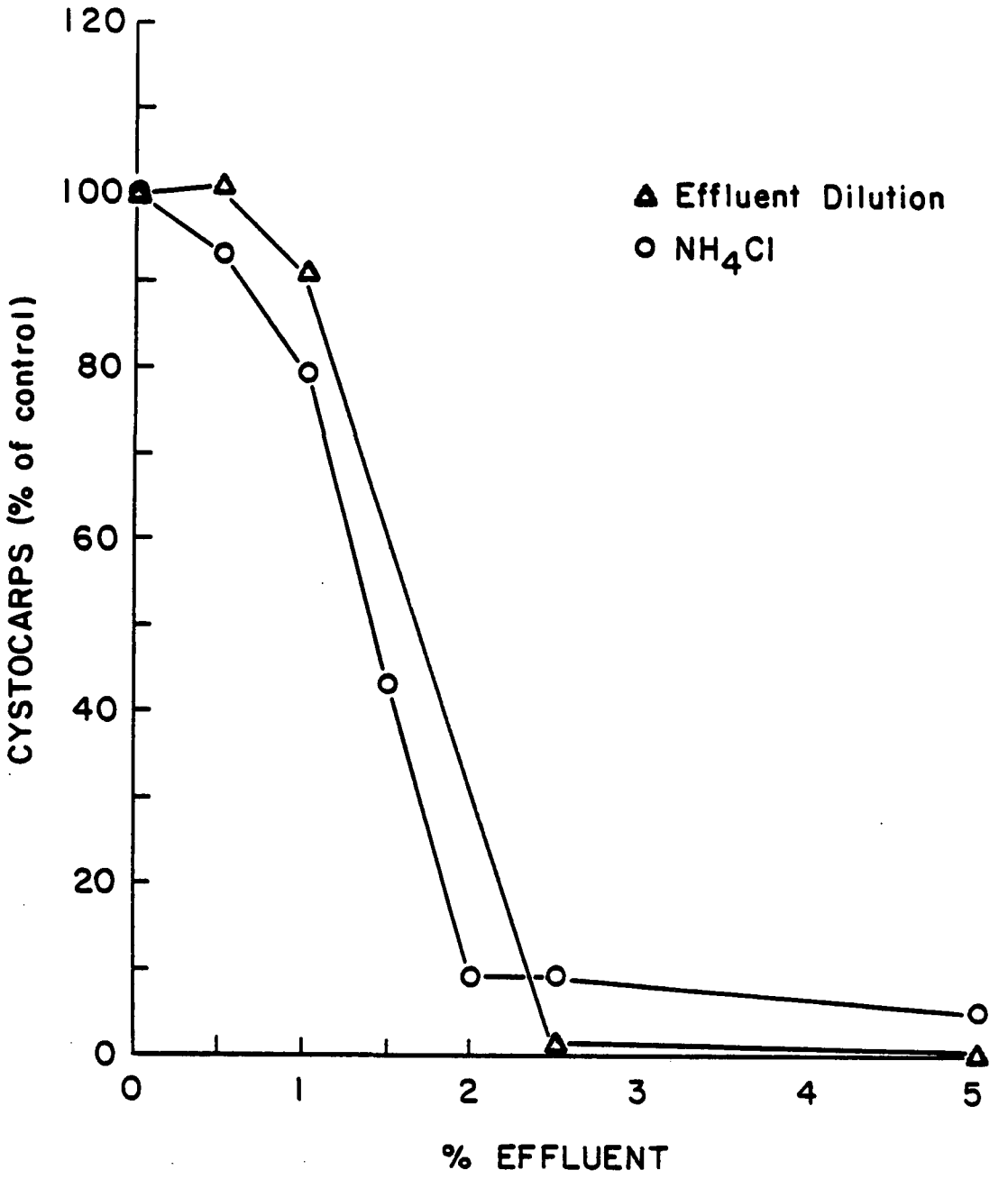


Figure 2. Number of cystocarps (as % of control) plotted against % effluent. The effluent data are averages from May 17th and 18th. The ammonium chloride data are based on 70 mg NH_4-N/L in the effluent. A stock solution of 26.7 mg NH_4Cl/100 mL was used.

PART IV

PRELIMINARY GUIDANCE MANUAL FOR CONDUCTING SEXUAL REPRODUCTION TESTS WITH THE MARINE MACROALGA, *LAMINARIA SACCHARINA*, FOR USE IN TESTING COMPLEX EFFLUENTS

INTRODUCTION

A test was developed to use kelp, Laminaria saccharina, as a toxicity test species for short-term exposure to toxicants. A test has also been developed using the red alga Champia parvula, however, this alga is primarily a warm water species. The kelps are more normally found in colder waters, and thus would make a good complimentary test organism. As with Champia, sexual reproduction was used as the endpoint for accessing the effects of toxicants with Laminaria. The test differs from that of Champia in that it requires preconditioning in order to perform tests.

The current Laminaria test is based on techniques that were first used with oil studies several years ago (Steele and Hanisak, 1979). Those tests used material derived from nature for each experiment, hereas the present test arelies on cultured gametophytic material.

MAINTENANCE OF STOCK CULTURES OF *LAMINARIA SACCHARINA*

Laminaria's life history is an alternation of microscopic gametophytes with a large diploid blade. The clones presently being used were isolated from Rhode Island in 1985. It is probably not essential to have a standard clone, however, some experience is required to isolate new clones from the field into unialgal culture. Unialgal stock cultures are necessary to maintain healthy, actively growing plants for use in testing.

Male and female plants of *Laminaria* can be maintained easily in unialgal culture in the laboratory. New cultures can be started by blending old cultures, and splitting into several new culture vessels. Blending is accomplished using a food blender at its fastest speed (for approximately 1 min). For maintenance cultures, a nutrient medium without added iron is used to inhibit gametogenesis; allowing greater vegetative growth. Some preconditioning is required to induce reproduction. Under the conditons listed below, male and female gametophytes will produce gametes. Thus, plant material can be available at any time for testing.

Unialgal stock cultures of both males and females are maintained in separate, aerated 500 mL Erlenmeyer flasks containing 400 mL of the culture medium. The choice of these flasks is one of preference rather than necessity. The maintenance culture medium is artificial or natural seawater to which additional nutrients are added (Table 1). Seawater is autoclaved for 30 min at 15 psi. The culture flasks are capped with aluminum foil and autoclaved dry, for 10 min. Culture medium is made up by dispensing seawater into the sterile flasks and adding the appropriate nutrients from a sterile stock solution. Alternately, 500 mL

flasks could be autoclaved with the seawater already in them. Sterilization is used to prevent microalgal contamination, and not to keep cultures bacteria-free.

We recommend that several cultures of both males and females be maintained simultaneously to keep a constant supply of plant material available. Initial stock cultures should be started weekly or biweekly with freshly blended material. Cultures are gently aerated through sterile, cotton-plugged, disposable, polystyrene 1 mL pipets. Cultures are capped with foam plugs and aluminum foil and illuminated with ca 75 uE m^{-2} s^{-1} of cool-white fluorescent light on a 16:8, light:dark cycle. The temperature is 12 to 15 °C and the salinity 28 to 30°/$_{oo}$. Media are changed biweekly. About one-half to one-third of the plant material should be placed into another culture vessel with each medium change to reduce the amount of biomass as the plants grow. With the above procedure, actively growing plants will be continuously available. The total number of cultures maintained will depend on the expected frequency of testing. We keep 7 actively growing cultures of each sex. In this way we can use a different culture each day and not reuse it for at least a week; allowing ample time for regrowth of the blended material.

ARTIFICIAL SEAWATER

Because salinity adjustments will be necessary in testing most complex effluents (particularly their receiving waters), an artificial seawater recipe that yields good growth of Laminaria is desirable. An artificial seawater would also make the test method more readily available to laboratories that do not have access to clean natural seawater. The recipe for the artificial seawater that we use is listed in Table 2. Comparisons between the sensitivity to effluents in this medium with

that in natural seawater have not yet been conducted.

PREPARATION OF PLANTS FOR A TEST

Stock cultures should be checked for their readiness for use in toxicity tests. Plants can be checked by examination under a compound microscope (50-100X). Healthy gametophytes of both sexes are highly branched and the condition of individual cells can be accessed by observing the chromatophores. These should appear as discrete pale brown discoid objects evenly dispersed in the cell. Senescent cells will appear much darker and the chromatophores will not be discrete. Once cultures are determined to be usable for toxicity testing, plants should be blended using a commercial food blender at the highest speed. The resulting suspension should be filtered through a 30-60 um nylon screening, and the portion that passes through used for testing. These cells can be diluted and pipetted into the test chambers.

TEST CHAMBERS

Most testing to date has been performed in ca. 30 mL (using 60x25mm petri dishes). These dishes offer the advantage of being disposable, as well as being deeper than the standard petri dish (Lab Tek #4036). Recovery chambers can be either these dishes or scintillation vials.

TEST CONDITIONS

Temperature, Salinity, Light and Aeration

The test exposure duration is 2 days followed by a 3-7 day recovery period for females for the development of sporophytes. The exposure temperature should be between 10-12°C, and the salinity should be between 28-32°/$_{oo}$. For receiving waters, salinity will often be below the desired range and must be adjusted with artificial sea salts. The

photoperiod should be a 16h:8h, light:dark cycle of ca. 75 uE m^{-2} s^{-1} of cool-white fluorescent light. Plants are recovered under the same salinity and light conditions, but the temperature is raised to 16°C to slow the growth of the females while the sporophytes develop (this is not essential, but it makes the counting of the sporophytes easier).

Nutrient

The nutrient recipe is listed in Table 1. Trace metals and EDTA are omitted from the medium during test exposures (except in artificial seawater, where EDTA is added at 20 ug/L).

PROTOCOL

1. At least one week prior to testing, blend males, filter and dilute and allow to settle onto cover slips (100-200/cover slip). The minus iron medium is used at this point. This allows the males to attach better to the cover slip before gametogenesis begins.
2. After 2-3 days replace medium for males with one that contains double strength iron/EDTA.
3. On the same day as #2, blend the females, filter and dilute and allow to settle onto cover slips or small pieces of glass slides (100-200/slide). The complete medium containing double strength iron is used.
4. After an additional 4-5 days check males and females for the presence of gametes. If gametes are seen then testing can begin.
5. Set up and label control and treatment dishes; three per treatment and controls.
6. Fill dishes with 30 mL of control or treatment water. Use the 1/8 strength nutrient medium minus trace elements and with the adjusted EDTA.
7. Add one cover slip (or glass slide) each of males and females.

Rinse males briefly in seawater to remove loosely attached plants. This will minimize the transfer of males to the slide containing the females.

8. After two days, remove slides containing females, rinse briefly in seawater and place into control medium (complete nutrients). Transfer to 16-18°C if possible.

9. After an additional 3-7 days examine females under a compound microscope and count the number of sporophytes.

10. Data are analyzed by analysis of variance (ANOVA) followed by Dunnett's mean separation test to determine differences from the control.

NOTE: We recommend making up four nutrient solutions.

1. Complete minus iron; for maintaining stock cultures.
2. complete plus iron; for recovery medium after exposure.
3. 1/8 strength minus trace elements and EDTA; for the exposure medium.
4. an iron/EDTA solution for increasing the iron concentration during gametogenesis.

Table 1. Recipe of nutrients to be added to artificial or natural seawater for stock cultures and test medium. The test medium concentrations are 1/8 strength of the stock culture concentration except for EDTA. The concentrated stock solution is autoclaved at standard temperature and pressure for 15 min (the pH is adjusted to 2.0 with HCl prior to autoclaving to prevent precipitation).

COMPOUND	AMOUNT/LITER STOCK CULTURES	CONCENTRATED STOCK[a]
$NaNO_3$	127 mg	12.7 g
$NaH_2PO_4 \cdot H_2O$	12.8 mg	1.28 g
$Na_2EDTA \cdot 2H_2O$	2.66 mg[b]	266 mg
$NaC_6H_5O_7$ (citrate)	1.03 mg	103 mg
Iron[c]	195 ug	19.5 mg
Trace Elements[d]		
$Na_2MoO_4 \cdot 2H_2O$	24.2 ug	2.42 mg
KI	83 ug	8.3 mg
$ZnSO_4 \cdot 7H_2O$	21.8 ug	2.18 mg
$NaVO_3$	6.1 ug	0.61 mg
$MnSO_4 \cdot H_2O$	0.61 ug	61.0 ug
Vitamins		
Thiamine·HCl	1.95 mg	
Biotin	1.0 ug	10 mL[e]
B_{12}	1.0 ug	

[a]Use 1.25 mL/L for test medium (with the adjusted EDTA concentration) and 10 mL/L for stock cultures. For test media an additional 2.5 mL/L of the sodium bicarbonate stock solution is added (see Table 3).

[b]The concentration is 20 ug/L for final solution of test media in artificial seawater; EDTA is omitted entirely if this nutrient medium is used in natural seawater.

[c]Iron stock solution prepared by dissolving 1 g iron powder in 10 mL concentrated HCl and diluting to 1 liter with deionized water. Acceptable stock solutions could also be made with ferric or ferrous chloride. Iron is omitted for maintenance culture medium and is double for initiation of gametes.

[d]Trace elements are omitted for toxicity test medium.

[e]Vitamin stock solution autoclaved separately in 10 mL sub-samples. Each 10 mL contains 195 mg of thiamine·HCl, 100 ug biotin, and 100 ug B_{12}. Adjust pH to ca 4.0 before autoclaving for 2 min.

Table 2. Recipe for artificial seawater using GP2. The concentrations of the salts have been adjusted to give a final salinity of 30°/₀₀. The original recipe calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), then all of the 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 should be for at least 10 min for 1 liter batches and 20 min for 10 to 20 liter volumes (at standard temperature and pressure).

COMPOUND	GRAMS/LITER[a]
NaCl	21.03
Na_2SO_4	3.52
KCl	0.61
KBr	0.088
$Na_2B_4O_7 \cdot 10H_2O$	0.034
$MgCl_2 \cdot 6H_2O$	9.50
$CaCl_2 \cdot 2H_2O$	1.32
$SrCl_2 \cdot 6H_2O$	0.02
$NaHCO_3$[b]	0.17

[a]Generally made in 10 to 20L batches.

[b]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.

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PART V

COMPARISON OF THE SENSITIVITY OF *LAMINARIA* WITH THAT OF *CHAMPIA*

INTRODUCTION

At this point, Laminaria looks very promising as a toxicity test species for testing single compounds and complex effluents. However, a comparison of test results using Laminaria with that of the test using Champia was necessary to determine the sensitivity of the new procedure to toxicants. The comparison was made using two heavy metals (silver and copper); two organics (pentachlorophenol and isophorone); and one sewage effluent (East Greenwich STP).

MATERIALS AND METHODS

The procedures used for both Laminaria and Champia are describe elsewhere in this report (Parts IV and I respectively).

RESULTS

The results of the comparison of Champia and Laminaria are shown in Figures 1 and 2 and Table 1. The sensitivity of Laminaria to copper and silver was not as great as that for Champia (Figure 1). In fact, Champia was at least an order of magnitude more sensitive. However, Laminaria and Champia were very similar in their sensitivities to both of the organics tested (Figure 2) and the sewage effluent (Table 1).

The results of these early comparisons indicate that Laminaria will be a useful toxicity test species. It was noteworthy that these two algal species, which come from vastly different phyla, responded similarly to the organics and sewage effluent. The gives support to the use of either species to make preliminary generalizations about the response of seaweeds to toxicants.

Table 1. The effect of sewage effluent from the East Greenwich STP on sexual reproduction in *Laminaria saccharina* and *Champia parvula*. The values listed are the geometric mean (as percent effluent) of the effect/no effect concentrations using no sexual reproduction as the effect measured.

Date	*Laminaria*	*Champia*
September 9, 1986	1.8	3.7
September 15, 1986	3.7	1.8

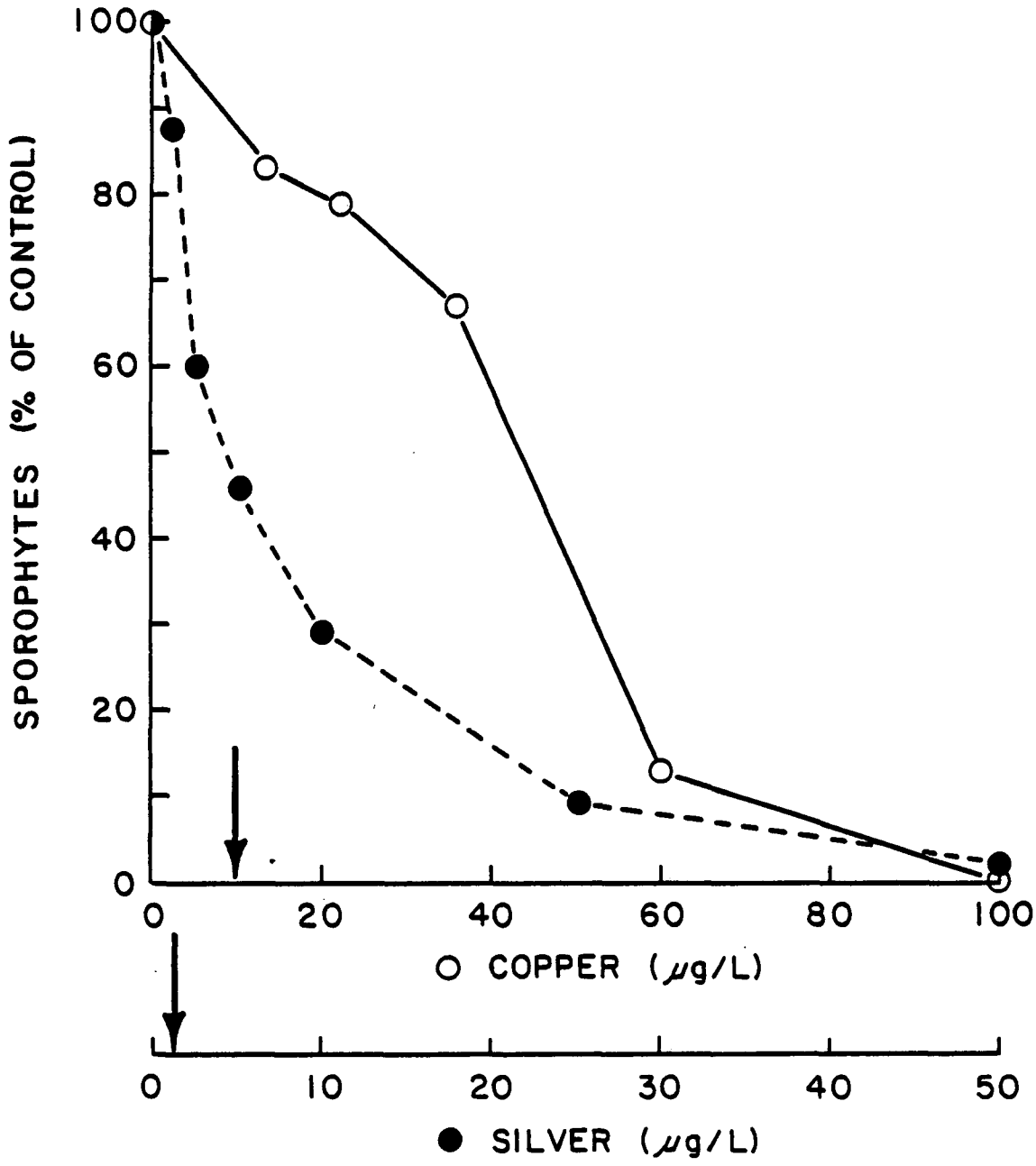

Figure 1. The effect of copper and silver on sexual reproduction in Laminaria saccharina and Champia parvula. The effect for Champia is represented only by an arrow where the total absence of reproduction occurred.

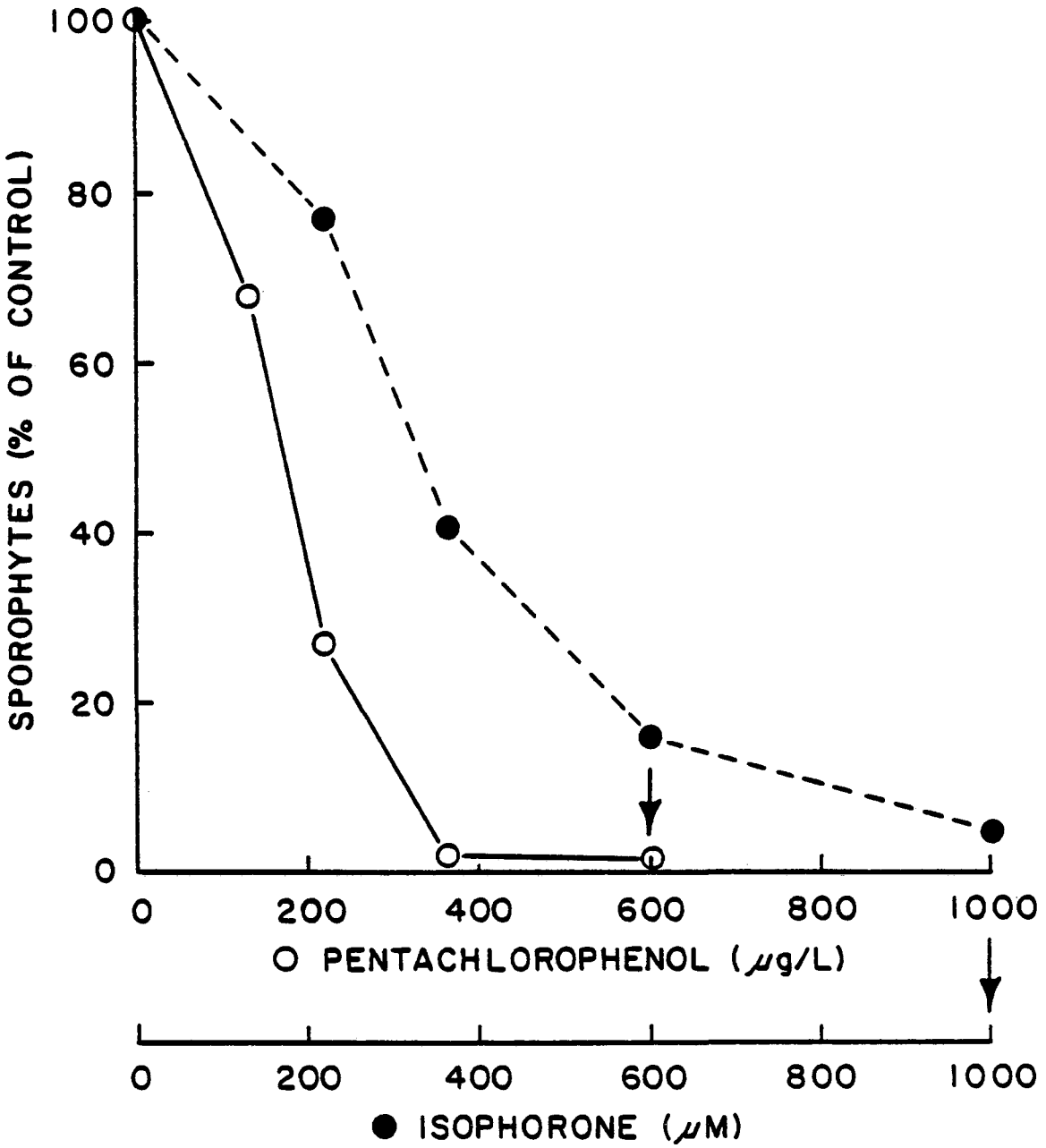

Figure 2. The effect of pentachlorophenol and isophorone on sexual reproduction in Laminaria saccharina and Champia parvula. The effect for Champia is represented only by an arrow where the absence of reproduction occurred.

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(Please read Instructions on the reverse before completing)

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Techniques developed during this cooperative agreement were presented at three Effluent Testing Workshops sponsored by ERLN in 1985 and 1986.

16. ABSTRACT

A previous sexual reproduction toxicity test with the marine red macroalga, Champia parvula, lasted two weeks. This report covers a modification to this procedure in which males and females are exposed together to a toxicant or effluent for only two days. The procedure was used successfully with single compounds and a variety of complex effluents. A comparison of toxicity test results between the two-week and the two-day exposure procedures was performed using single compounds. The two procedures compared favorably. Initial steps were also taken in the development of a similar two-day exposure test with the brown alga, Laminaria saccharine. Laminaria represent another phylum of algae than Champia, and has the additional advantage of being both economically and ecologically important. For the current study, the feasibility of using Laminaria as a routine toxicity test species was verified. A comparison was also made between the sensitivity of both species. The sensitivity of Laminaria to copper and silver was not as great as that for Champia. However, both species were very similar in their response to pentachlorophenol and isophorone, as well as a sewage effluent.

17. KEY WORDS AND DOCUMENT ANALYSIS

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Champia, Laminaria effluents		

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