SECTION 17

GIANT KELP, *Macrocystis pyrifera* GERMINATION AND GERM-TUBE GROWTH TEST METHOD

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GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GROWTH TEST

17.1 SCOPE AND APPLICATION

17.1.1 This method estimates the chronic toxicity of effluents and receiving water to zoospores and embryonic gametophytes of giant kelp, *Macrocystis pyrifera* during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

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

17.1.3 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 and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

17.1.4 This method is commonly used in one of two forms: (1) a definitive test, consisting of 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.

17.1.5 This method should be restricted to use by, or under the supervision of, professionals experienced in aquatic toxicity testing. Specific experience with any toxicity test is usually needed before acceptable results become routine.

17.2 SUMMARY OF METHOD

17.2.1 This method provides step-by-step instructions for performing a 48-h day static non-renewal toxicity test using giant kelp to determine the toxicity of substances in marine and

estuarine waters. The test endpoints are germination of gameophyte spores and length of embryonic gametophyte germination tubes.

17.3 **INTERFERENCES**

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

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

17.4 **SAFETY**

17.4.1 See Section 3, Health and Safety.

17.5 **APPARATUS AND EQUIPMENT**

17.5.1 Tanks, trays, or aquaria -- for holding and acclimating giant kelp, e.g., standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 10-20EC), with appropriate filtration and aeration system.

17.5.2 Air pump, air lines, and air stones -- for aerating water containing broodstock or for supplying air to test solutions with low dissolved oxygen.

17.5.3 Constant temperature chambers or water baths -- for maintaining test solution temperature and keeping dilution water supply, gametes, and embryo stock suspensions at test temperature (15EC) prior to the test.

17.5.4 Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.

17.5.5 Refractometer -- for determining salinity.

17.5.6 Hydrometer(s) -- for calibrating refractometer.

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

17.5.8 Thermometer, National Bureau of Standards Certified (see USEPA METHOD l70.l, USEPA, l979) -- to calibrate laboratory thermometers.

17.5.9 pH and DO meters -- for routine physical and chemical measurements.

17.5.10 Standard or micro-Winkler apparatus -- for determining DO (optional) and calibrating the DO meter.

17.5.11 Winkler bottles -- for dissolved oxygen determinations.

17.5.12 Balance -- Analytical, capable of accurately weighing to 0.0001 g.

17.5.13 Fume hood -- to protect the analyst from effluent or formaldehyde fumes.

17.5.14 Glass stirring rods -- for mixing test solutions.

17.5.15 Graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-l000 mL for making test solutions. (Note: not to be used interchangeably for gametes or embryos and test solutions).

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

17.5.17 Pipets, automatic -- adjustable, to cover a range of delivery volumes from 0.010 to 1.000 mL.

17.5.18 Pipet bulbs and fillers -- PROPIPET® or equivalent.

17.5.19 Wash bottles -- for reagent water, for topping off graduated cylinders, for rinsing small glassware and instrument electrodes and probes.

17.5.20 Wash bottles -- for dilution water.

17.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

17.5.22 Cubitainers, beakers, or similar chambers of non-toxic composition for holding, mixing, and dispensing dilution water and other general non-effluent, non-toxicant contact uses. These should be clearly labeled and not used for other purposes.

17.5.23 Beakers, 250 borosilicate glass -- for mixing test solutions.

17.5.24 Beakers, 1,000 mL borosilicate glass -- for holding sporophyll blades.

17.5.25 Inverted or compound microscope -- for inspecting zoopspores and embryonic gametophytes.

17.5.26 Hemacytometer (bright-line rbc) -- for measuring zoospore density.

17.5.27 Counter, two unit, 0-999 -- for recording counts of zoopspores.

17.5.28 Light meter (irradiance meter w/cosine corrected sensor) -- for measuring light intensity.

17.5.29 Cool white fluorescent lights -- for providing light during incubation of developing gametophytes.

17.5.30 60 µm NITEX® filter -- for filtering receiving water.

17.6 **REAGENTS AND SUPPLIES**

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

17.6.2 Data sheets (one set per test) -- for data recording (Figures 1 and 2).

17.6.3 Tape, colored -- for labelling test chambers and containers.

17.6.4 Markers, water-proof -- for marking containers, etc.

17.6.5 Parafilm -- to cover graduated cylinders and vessels.

17.6.6 Gloves, disposable -- for personal protection from contamination.

17.6.7 Pipets, serological -- l-l0 mL, graduated.

17.6.8 Pipet tips -- for automatic pipets.

17.6.9 Coverslips -- for microscope slides.

17.6.10 Lens paper -- for cleaning microscope optics.

17.6.11 Laboratory tissue wipes -- for cleaning and drying electrodes, microscope slides, etc.

17.6.12 Disposable countertop covering -- for protection of work surfaces and minimizing spills and contamination.

17.6.13 pH buffers 4, 7, and l0 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method l50.l, USEPA, l979).

17.6.14 Membranes and filling solutions -- for dissolved oxygen probe (see USEPA Method 360.l, USEPA, l979), or reagents for modified Winkler analysis.

17.6.15 Laboratory quality assurance samples and standards -- for the above methods.

17.6.16 Test chambers -- 600 mL, five chambers per concentration. The chambers should be borosilicate glass (for effluents) or nontoxic disposable plastic labware (for reference toxicants). To avoid contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered during the test with safety glass plates or a plastic sheet (6 mm thick).

17.6.17 Glutaraldehyde -- for specimen preservation - optional; (see Section 17.10.8.2).

17.6.18 Microscope slide (flat) -- for each test chamber to serve as the substratum upon which the zoospores will settle.

17.6.19 Reference toxicant solutions (see Section 17.10.2.4 and see Section 4, Quality Assurance).

17.6.20 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 and Section 7, Dilution Water).

17.6.21 Effluent and receiving water -- see Section 8, Effluent and Surface Water Sampling, and Sample Handling, and Sample Preparation for Toxicity Tests.

17.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 17.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

17.6.23 HYPERSALINE BRINES

17.6.23.1 Most industrial and sewage treatment effluents entering marine and estuarine systems have little measurable salinity. Exposure of larvae to these effluents will usually require increasing the salinity of the test solutions. It is important to maintain an essentially constant salinity across all treatments. In some applications it may be desirable to match the test salinity with that of the receiving water (See Section 7.1). Two salt sources are available to adjust salinities -- artificial sea salts and hypersaline brine (HSB) derived from natural seawater. Use of artificial sea salts is necessary only when high effluent concentrations preclude salinity adjustment by HSB alone.

17.6.23.2 Hypersaline brine (HSB) can be made by concentrating natural seawater by freezing or evaporation. HSB should be made from high quality, filtered seawater, and can be added to the effluent or to reagent water to increase salinity. HSB has several desirable characteristics for use in effluent toxicity testing. Brine derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the

microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and it can be stored for prolonged periods without any apparent degradation. However, even if the maximum salinity HSB (100‰) is used as a diluent, the maximum concentration of effluent (0‰) that can be tested is 66% effluent at 34‰ salinity (see Table 1).

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED AT 34‰ WITHOUT THE ADDITION OF DRY SALTS GIVEN THE INDICATED EFFLUENT AND BRINE SALINITIES.

Effluent Salinity ‰	Brine 60 ‰	Brine 70 ‰	Brine 80 ‰	Brine 90 ‰	Brine 100 ‰
0	43.33	51.43	57.50	62.22	66.00
1	44.07	52.17	58.23	62.92	66.67
2	44.83	52.94	58.97	63.64	67.35
3	45.61	53.73	59.74	64.37	68.04
4	46.43	54.55	60.53	65.12	68.75
5	47.27	55.38	61.33	65.88	69.47
10	52.00	60.00	65.71	70.00	73.33
15	57.78	65.45	70.77	74.67	77.65
20	65.00	72.00	76.67	80.00	82.50
25	74.29	80.00	83.64	86.15	88.00

17.6.23.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the freezer or the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

17.6.23.4 Freeze Preparation of Brine

17.6.23.4.1 A convenient container for making HSB by freezing is one that has a bottom drain. One liter of brine can be made from

four liters of seawater. Brine may be collected by partially freezing seawater at -10 to -20EC until the remaining liquid has reached the target salinity. Freeze for approximately six hours, then separate the ice (composed mainly of fresh water) from the remaining liquid (which has now become hypersaline).

17.6.23.4.2 It is preferable to monitor the water until the target salinity is achieved rather than allowing total freezing followed by partial thawing. Brine salinity should never exceed 100‰. It is advisable not to exceed about 70‰ brine salinity unless it is necessary to test effluent concentrations greater than 50%.

17.6.23.4.3 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.5 Heat Preparation of Brine

17.6.23.5.1 The ideal container for making brine using heat-assisted evaporation of natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method is to use a thermostatically controlled heat exchanger made from fiberglass. If aeration is needed, use only oil-free air compressors to prevent contamination.

17.6.23.5.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough reagent water rinses.

17.6.23.5.3 Seawater should be filtered to at least 10 µm before being put into the brine generator. The temperature of the seawater is increased slowly to 40EC. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

17.6.23.5.4 After the required salinity is attained, the HSB should be filtered through a 1 µm filter and poured directly into portable containers (20-L cubitainers or polycarbonate water cooler jugs are suitable). The brine storage containers should be capped and labelled with the salinity and the date the brine was generated. Containers of HSB should be stored in the dark at 4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

17.6.23.6 Artificial Sea Salts

17.6.23.6.1 No data from giant kelp tests using sea salts or artificial seawater (e.g., GP2) are available for evaluation at this time, and their use must be considered provisional.

17.6.23.7 Dilution Water Preparation from Brine

17.6.23.7.1 Although salinity adjustment with brine is the preferred method, the use of high salinity brines and/or reagent water has sometimes been associated with discernible adverse effects on test organisms. For this reason, it is recommended that only the minimum necessary volume of brine and reagent water be used to offset the low salinity of the effluent, and that brine controls be included in the test. The remaining dilution water should be natural seawater. Salinity may be adjusted in one of two ways. First, the salinity of the highest effluent test concentration may be adjusted to an acceptable salinity, and then serially diluted. Alternatively, each effluent concentration can be prepared individually with appropriate volumes of effluent and brine.

17.6.23.7.2 When HSB and reagent water are used, thoroughly mix together the reagent water and HSB before mixing in the

effluent. Divide the salinity of the HSB by the expected test salinity to determine the proportion of reagent water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 34‰, 100‰ divided by 34‰ = 2.94. The proportion of brine is 1 part, plus 1.94 parts reagent water. To make 1 L of dilution water at 34‰ salinity from a HSB of 100‰, 340 mL of brine and 660 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

17.6.23.8 Test Solution Salinity Adjustment

17.6.23.8.1 Table 2 illustrates the preparation of test solutions (up to 50% effluent) at 34‰ by combining effluent, HSB, and dilution water. Note: if the highest effluent concentration does not exceed 50% effluent, it is convenient to prepare brine so that the sum of the effluent salinity and brine salinity equals 68‰; the required brine volume is then always equal to the effluent volume needed for each effluent concentration as in the example in Table 2.

17.6.23.8.2 Check the pH of all test solutions and adjust to within 0.2 units of dilution water pH by adding, dropwise, dilute hydrochloric acid or sodium hydroxide (see Section 8.8.9, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

17.6.23.8.3 To calculate the amount of brine to add to each effluent dilution, determine the following quantities: salinity of the brine (SB, in ‰), the salinity of the effluent (SE, in ‰), and volume of the effluent to be added (VE, in mL). Then use the following formula to calculate the volume of brine (VB, in mL) to be added:

$$VB = VE \times (34 - SE)/(SB - 34)$$

17.6.23.8.4 This calculation assumes that dilution water salinity is 34 ± 2‰.

17.6.23.9 Preparing Test Solutions

17.6.23.9.1 Two hundred mL of test solution are needed for each test chamber. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution

water. For example, to prepare 1% effluent, add 10 mL of effluent to a 1-liter volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 1-Liter mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

17.6.23.9.2 To prepare a test solution at higher effluent concentrations, hypersaline brine must usually be used. For example, to prepare 40% effluent, add 400 mL of effluent to a 1-liter volumetric flask. Then, assuming an effluent salinity of 2‰ and a brine salinity of 66‰, add 400 mL of brine (see equation above and Table 2) and top off the flask with dilution water. Stopper the flask and shake well. Distribute equal volumes into the replicate test chambers.

17.6.23.10 Brine Controls

17.6.23.10.1 Use brine controls in all tests where brine is used. Brine controls contain the same volume of brine as does the highest effluent concentration using brine, plus the volume of reagent water needed to reproduce the hyposalinity of the effluent in the highest concentration, plus dilution water. Calculate the amount of reagent water to add to brine controls by rearranging the above equation, (See, 17.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 34)/(34 - SE)$$

If effluent salinity is essentially 0‰, the reagent water volume needed in the brine control will equal the effluent volume at the highest test concentration. However, as effluent salinity and effluent concentration increase, less reagent water volume is needed.

17.6.24 TEST ORGANISMS

17.6.24.1 The test organisms for this method are the zoospores of the giant kelp, *Macrocystis pyrifera*. *Macrocystis* is the dominant canopy forming Laminarian alga in southern and central California and forms extensive subtidal forests along the coast. Giant kelp forests support a rich diversity of marine life and provide habitat and food for hundreds of invertebrate and vertebrate species (North, 1971; Foster and Schiel, 1985). It

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (x‰), BRINE, AND DILUTION WATER NEEDED FOR ONE LITER OF EACH TEST SOLUTION.

FIRST STEP: Combine brine with reagent water or natural seawater to achieve a brine of 68-x‰ and, unless natural seawater is used for dilution water, also a brine-based dilution water of 34‰.

SERIAL DILUTION:

Step 1. Prepare the highest effluent concentration to be tested by adding equal volumes of effluent and brine to the appropriate volume of dilution water. An example using 40% is shown.

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	800 mL	800 mL	400 mL

Step 2. Use either serially prepared dilutions of the highest test concentration or individual dilutions of 100% effluent.

Effluent Conc. (%)	Effluent Source	Dilution Water* (34‰)
20	1000 mL of 40%	1000 mL
10	1000 mL of 20%	1000 mL
5	1000 mL of 10%	1000 mL
2.5	1000 mL of 5%	1000 mL
Control	none	1000 mL

INDIVIDUAL PREPARATION

Effluent Conc. (%)	Effluent x‰	Brine (68-x)‰	Dilution Water* 34‰
40	400 mL	400 mL	200 mL
20	200 mL	200 mL	600 mL
10	100 mL	100 mL	800 mL
5	50 mL	50 mL	900 mL
2.5	25 mL	25 mL	950 mL
Control	none	none	1000 mL

*May be natural seawater or brine-reagent water equivalent.

is an appropriate toxicity test species because of its availability, economic and ecological importance, history of successful laboratory culture (North, 1976; Luning, 1980; Kuwabara, 1981; Deysher and Dean, 1984; Linfield, 1985), and previous use in toxicity testing (Smith and Harrison, 1978; James et al., 1987; Anderson and Hunt, 1988; Hunt et al., 1989; Anderson et al., 1990). Other Laminarian alga species have proven to be useful for laboratory toxicity testing (Chung and Brinkhuis, 1986; Thompson and Burrows, 1984; Hopkin and Kain, 1978; see Thursby et al., 1993 for review).

17.6.24.2 Like all kelps, *Macrocystis* has a life cycle that alternates between a microscopic gametophyte stage and a macroscopic sporophyte stage. It is the sporophyte stage that forms kelp forests. These plants produce reproductive blades (sporophylls) at their base. The sporophylls develop patches (sori) in which biflagellate, haploid zoospores are produced. The zoospores are released into the water column where they swim and eventually settle onto the bottom and germinate. The dioecious spores develop into either male or female gametophytes. The male gametophytes produce flagellated gametes which may fertilize eggs produced by the female gametophytes. Fertilized eggs develop into sporophytes within 12- 15 days, completing the lifecycle.

17.6.24.3 The method described here focuses on germination of the zoospores and the initial growth of the developing gametophytes. It involves the controlled release of zoospores from the sporophyll blades, followed by the introduction of a spore suspension of known density into the test containers. The zoospores swim through the test solution and eventually settle onto glass microscope slides. The settled spores germinate by extruding the cytoplasm of the spore through the germ-tube into the first gametophytic cell. This stage is often referred to as the "dumbell" stage. The two endpoints measured after 48 hours are germination success and growth of the embryonic gametophytes (germ-tube length).

17.6.24.4 Species Identification

17.6.24.4.1 Although there is some debate over the taxonomy of the genus *Macrocystis*, Abbott and Hollenberg (1976) consider only two species in California: *M. pyrifera*, and *M. integrifolia*. The

two are distinguished from each other based on habitat and the morphology of their holdfasts. *Macrocystis pyrifera* occurs subtidally while *M. integrifolia* occurs in the low intertidal and shallow subtidal zones. *Macrocystis pyrifera* has a conical holdfast while *M. integrifolia* has a more flattened, creeping holdfast. Consult Abbott and Hollenberg (1976) for a more detailed taxonomic discussion of the two species.

17.6.24.5 Obtaining Zoospores

17.6.24.5.1 *Macrocystis* zoospores are obtained from the reproductive blades (sporophylls) of the adult plant. The sporophylls are located near the base of the plant just above its conical holdfast. Sporophylls must be collected subtidally and should be collected from at least five different plants in any one location to give a good genetic representation of the population. The sporophylls should be collected from areas free of point and non-point source pollution to minimize the possibility of genetic or physiological adaptation to pollutants. In situations where a thermocline is present at the collection site, the sporophylls should be collected from below the thermocline to ensure adequate spore release. Sporophylls are identified in the field by the presence of darkened patches called sori. The zoospores develop within the sori. In addition, the sporophylls are distinguished from vegetative blades by their thinner width, basal location on the adult plant, and general lack of pneumatocysts (air bladders). Collection of algae is regulated by California law. Collectors must obtain a scientific collector's permit from the California Department of Fish and Game and observe any regulations regarding collection and transport of kelp. For further information regarding sporophyll collection, contact the Marine Pollution Studies Laboratory, 34500 Coast Route 1, Granite Canyon, Monterey CA, 93940, (408) 624-0947.

17.6.24.6 Broodstock Culture and Handling

17.6.24.6.1 After collection, the sporophylls should be kept damp and not exposed to direct sunlight. Avoid immersing the blades in seawater, however, to prevent premature spore release. The sporophylls should be <u>rinsed thoroughly</u> in 0.2 µm filtered seawater to remove diatoms and other epiphytic organisms. The individual blades can be gently rubbed between fingers under

running filtered seawater or brushed with a soft bristled brush. The blades are stored between moist paper towels (lasagna style so that the sporophylls do not overlap each other, and each layer of sporophylls are separated by a layer of paper towels) at approximately 9-12EC until needed. The zoospores must be released within 24 hours of collection to insure their viability. Preliminary data indicate that prolonged storage times may affect test results (Bottomley et al., 1991); however as long as germination rates meet control acceptability criteria this should not affect test results. Sporophylls should be kept shaded to prevent damage to the spores. For holding or transport times longer than approximately six hours, the sporophylls should be placed in an ice chest with blue ice. The blue ice should be wrapped in newspaper (10 layers) for insulation, then plastic to prevent leaking.

17.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

17.8 CALIBRATION AND STANDARDIZATION

17.8.1 See Section 4, Quality Assurance.

17.9 QUALITY CONTROL

17.9.1 See Section 4, Quality Assurance.

17.10 TEST PROCEDURES

17.10.1 TEST DESIGN

17.10.1.1 The test consists of at least five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain five replicates of a brine control.

17.10.1.2 Effluent concentrations are expressed as percent effluent.

17.10.2 TEST SOLUTIONS

17.10.2.1 Receiving waters

17.10.2.1.1 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 five replicate chambers per test, each containing 200 mL, analysis would require approximately 1 L of sample per test.

17.10.2.2 Effluents

17.10.2.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of at least 0.5 is commonly used. A dilution factor of 0.5 provides hypothesis test discrimination of ± 100%, and testing of a 16 fold range of concentrations. Hypothesis test discrimination shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **USEPA recommends that one of the five effluent treatments must be a concentration of effluent mixed with dilution water which corresponds to the permittee's instream waste concentration (IWC).** At least two of the effluent treatments must be of lesser effluent concentration than the IWC, with one being at least one-half the concentration of the IWC. If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 66% at 34‰ salinity.

17.10.2.2.2 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%).

17.10.2.2.3 The volume in each test chamber is 200 mL.

17.10.2.2.4 Effluent dilutions should be prepared for all replicates in each treatment in one container to minimize variability among the replicates. Dispense into the appropriate effluent test chambers.

17.10.2.3 Dilution Water

17.10.2.3.1 Dilution water should be uncontaminated 1-µm-

filtered natural seawater or hypersaline brine prepared from uncontaminated natural seawater plus reagent water (see Section 7, Dilution Water). Natural seawater may be uncontaminated receiving water. This water is used in all dilution steps and as the control water.

17.10.2.4 Reference Toxicant Test

17.10.2.4.1 Reference toxicant tests should be conducted as described in Quality Assurance (see Section 4.7).

17.10.2.4.2 The preferred reference toxicant for giant kelp is copper chloride ($CuCl_2 \circ 2H_2O$). Reference toxicant tests provide an indication of the sensitivity of the test organisms and the suitability of the testing laboratory (see Section 4 Quality Assurance). Another toxicant may be specified by the appropriate regulatory agency. Prepare a 10,000 µg/L copper stock solution by adding 0.0268 g of copper chloride ($CuCl_2 \circ 2H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

17.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 5.6, 10, 18, 32, 100, and 180 µg/L total copper. Prepare one liter of each concentration by adding 0, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, and 18.0 mL of stock solution, respectively, to one-liter volumetric flasks and fill with dilution water. Start with control solutions and progress to the highest concentration to minimize contamination.

17.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use zoospores from the same release. The tests must be handled in the same way and test solutions delivered to the test chambers at the same time. Reference toxicant tests must be conducted at 34 ± 2‰.

17.10.3 RELEASE OF ZOOSPORES FOR THE TEST

17.10.3.1 Zoospores are released by slightly desiccating the sporophyll blades, and then placing them in filtered seawater. To desiccate the sporophylls, blot the blades with paper towels and expose them to air for 1 hour.

17.10.3.2 The number of sporophyll blades needed depends upon their maturity; usually 25-30 blades (~ 100 grams wet weight) are sufficient. After 1 hour the blades should be rinsed again thoroughly using 0.2 µm-filtered seawater, then placed in a one L glass or plastic beaker filled with 0.2 µm filtered seawater at 15-16EC. The release water should never exceed 18EC.

17.10.3.3 After one hour, a sufficient number of zoospores should be present to conduct the test. The presence of zoospores is indicated by a slight cloudiness in the water. To verify whether zoospores are present, periodically sample the solution and observe the sample microscopically (100x).

17.10.3.4 To insure that the zoospores are viable and have not begun to germinate before they are exposed to the toxicant, the zoospore release process should not be longer than two hours. If it takes longer than two hours to get an adequate density of zoospores (~7,500 zoospores/mL of test solution), repeat the release process with a new batch of sporophylls.

17.10.3.5 After the zoospores are released, remove the sporophylls and let the spore mixture settle for 30 minutes. After 30 minutes, decant 250 mLs from the top of the spore solution into a separate clean glass beaker. Sample the spore solution and determine the spore density using a bright-line hemacytometer (100x). Spores may be counted directly, or to obtain a more accurate count, fix a sample of spores by mixing nine milliliters of spore solution with 1-mL of 37% buffered formalin (or acetic acid) in a test tube. Shake the sample well before placing it on the hemacytometer.

17.10.3.6 After counting, the density is multiplied by 1.111 to correct for the dilution caused by adding 1 mL of formalin to the sample. Use at least five replicate counts. After the density is determined, calculate the volume of zoospores necessary to give approximately 7,500 spores/mL of test solution. To prevent over-dilution of the test solution, this volume should not exceed 1% of the test solution volume. If this volume exceeds 1% of the test solution volume, it should be noted in the results.

17.10.3.7 Test solutions must be prepared while the zoospores are releasing from the sporophylls. Test solutions must be mixed, sampled, and temperature equilibrated in time to receive

the swimming zoospores as soon as they are counted. Zoospore release and counting should be done in a room separate from that used for toxicant preparation, and care should be taken to avoid contaminating the zoospores prior to testing.

17.10.4 START OF THE TEST

17.10.4.1 Prior to Beginning the Test

17.10.4.1.1 The test should begin as soon as possible, preferably within 24 h of sample collection. 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 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).

17.10.4.1.2 Just prior to test initiation (approximately 1 h), the temperature of a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (15 ± 1EC) and maintained at that temperature during the addition of dilution water.

17.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (15 ± 1EC).

17.10.4.1.4 Randomize the placement of test chambers in the temperature-controlled water bath, room, or incubator at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a random numbers or similar process (see Appendix A, for an example of randomization). Maintain the chambers in this configuration throughout the test, using a position chart. Record these numbers on a separate data sheet together with the concentration and replicate numbers to which they correspond. Identify this sheet with the date, test organism, test number, laboratory, and investigator's name, and safely store it away until after the gametophyte spores have been examined at the end of the test.

17.10.4.1.5 Note: Loss of the randomization sheet would invalidate the test by making it impossible to analyze the data afterwards. Make a copy of the randomization sheet and store

separately. Take care to follow the numbering system exactly while filling chambers with the test solutions.

17.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly and filled with test solutions, they can be arranged in

numerical order for convenience, since this will also ensure random placement of treatments.

17.10.4.2 Estimation of Zoospore Density

17.10.4.2.1 After determining the zoospore density and calculating the volume yielding 7,500 zoospores/mL test solution, add this volume to each test chamber (this is the start time of the test). Observe a sample of zoospores microscopically to verify that they are swimming before adding them to the test chambers.

17.10.4.2.2 Incubate the developing gametophytes for 48 hours in the test chambers at 15EC under 50 µE/m^2/s. The zoospores germinate and develop to the "dumbell" gametophyte stage during the exposure period.

17.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

17.10.5.1 The lights used in this method are cool white fluorescent lights adjusted to give 50 µE/m^2/s at the top of each test chamber. Each test chamber must receive the same quanta of light (50 ± 10 µE/m^2/s). Areas of increased light can be eliminated by taping the outside of the light diffuser or wrapping the fluorescent bulbs with aluminum foil.

17.10.5.2 The water temperature in the test chambers should be maintained at 15 ± 1EC. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be as deep as possible without floating the chambers.

15.10.5.3 The test salinity should be in the range of 34 ± 2‰. 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.

15.10.5.4 Rooms or incubators with high volume ventilation should be used with caution because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test chambers with clean polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

17.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

17.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentration should be measured on new solutions at the start of the test (Day 0). 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 that necessary to maintain a minimum acceptable DO and under no circumstances should it exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet No. 37033, or equivalent.

17.10.7 OBSERVATIONS DURING THE TEST

17.10.7.1 Routine Chemical and Physical Observations

17.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

17.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously or 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 the end of the test to determine temperature variation in the environmental chamber.

17.10.7.1.3 Record all the measurements on the data sheet.

17.10.8 TERMINATION OF THE TEST

17.10.8.1 Ending the Test

17.10.8.1.1 Record the time the test is terminated.

17.10.8.1.2 Temperature, pH, dissolved oxygen, and salinity are measured at the end of the exposure period in one test chamber at each concentration and in the control.

17.10.8.2 Sample preservation

17.10.8.2.1 In some cases it may be convenient to preserve the kelp cultures for later analysis. Preliminary work by Anderson and Hunt (Marine Pollution Studies Laboratory unpublished data) indicates that cultures can be preserved in 0.1% glutaraldehyde (final concentration) and that preservation has no significant effect on germination or germ-tube growth. Other researchers have used higher glutaraldehyde concentrations and found adequate preservation with no effect on spore germination or gametophyte growth (K. Goodwin, Calif. Inst. of Tech., unpublished data).

17.10.8.2.2 Because data on the effects of preservation are preliminary, it is recommended that anyone interested in preserving kelp cultures for later analysis first demonstrate that preservation does not affect test results. This can be accomplished by comparing germination and germ-tube growth in preserved vs non-preserved kelp cultures. We also recommend that if it is necessary to preserve kelp cultures for later analysis, a complete test should be preserved so that if any replicates are read preserved, all of the replicates should be read preserved. In the case where concurrent reference toxicant and complex effluent tests are conducted, it may be convenient to fix one test in glutaraldehyde and read the other test immediately.

17.10.8.2.3 When fixing kelp cultures, it is important to minimize disturbance to the gametophytes. Make sure that the culture slides are fixed and stored horizontally. We have used disposable petri dishes for preservation chambers; these allow individual replicate slides to be labelled and preserved separately to avoid mixing replicates. **Note:** Glutaraldehyde is toxic. If you intend to use this material as a preservative, study the material data safety sheets from the supplier and

follow strict safety precautions. Make sure test chambers and solutions contaminated with this material are disposed of properly.

17.10.8.3 Counting

17.10.8.3.1 After 48 hours, the test is terminated. Because it takes a considerable amount of time to read the test, reading can begin after 45 hours and must be completed within six hours. Remove the slide without decanting the test solution. The test slide can be lifted from the bottom of the test chamber with a separate clean microscope slide. Blot the bottom on a paper towel and place an 18-mm square cover slip on the slide. Blot the excess water around the edge of the cover slip to eliminate the flow of water under the cover slip.

17.10.8.4 Endpoints

17.10.8.4.1 The endpoints measured for the 48 hour *Macrocystis* method are percent germination success and germination tube length. Germination is considered successful if a germ-tube is present on the settled zoospore. Germination is considered to be unsuccessful if no germination tube is visible. To differentiate between a germinated and non-germinated zoospore, observe the settled zoospores at 400x magnification and determine whether they are circular (non-germinated) or have a protuberance that extends at least one spore diameter (about 3.0 µm) from the edge of the spore (germinated). Spores with a germination tubes less than one spore diameter are considered non-germinated.

17.10.8.4.2 The first 100 spores encountered while moving across the microscope slide are counted for each replicate of each treatment. **Note:** Sewage effluents may contain certain objects, such as ciliates, which look similar to non-germinated kelp spores. It is important to ensure that only kelp spores are counted for this endpoint. Kelp spores are green-brown in color, spherical, and lack mobility. Also, components of the cytoplasm of kelp spores appear to fluoresce a light green color when the spore is slightly out of focus. If a particular object cannot be identified, it should not be counted.

17.10.8.4.3 The growth endpoint is the measurement of the total length of the germination tube from the edge of the original

spore membrane. Only germinated spores with straight germination tubes and within the same focal plane are measured; if a spore is not completely in focus from tip to tip it should not be measured. The spores to be measured are randomly selected by moving the microscope stage to a new field of view without looking through the ocular lens.

17.10.8.4.4 Measure the germination-tube length of the spore whose spore case center is nearest the micrometer in each field; the spores case can be distinguished from the growing tip because it is usually clear (empty) at 48 hours, and it is more circular than the growing tip. If more than one spore case is touching the micrometer, both (or all) germinated spores are measured. A total of 10 spores for each replicate of each treatment are measured. It is easier to measure germ-tube length with a micrometer having a 10 mm linear scale (0.1 mm subdivisions); measure lengths to the nearest micron (typically to the nearest half micrometer unit; see Section 10200E, Standard Methods 17th edition, for micrometer/microscope calibration procedures). In situations where germination is significantly inhibited it may be difficult to find germinated spores for germ-tube growth measurement using the random search technique.

17.10.8.4.5 To expedite reading, the slide can be scanned to find germinated spores if germination is 30% or less. In this situation the first 10 spores encountered are measured for germ-tube length.

17.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

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

17.12 **ACCEPTABILITY OF TEST RESULTS**

17.12.1 For tests to be considered acceptable, the following requirements must be met:

(1) Mean control germination must be at least 70% in the controls.

(2) Mean germination-tube length in the controls must be at least 10 µm in the controls.

(3) The germination-tube growth NOEC must be below 35 µg/liter in the reference toxicant test.

(4) The minimum significant difference (%MSD) is <20% relative to the control for both germination and germ-tube length in the reference toxicant test.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR GIANT KELP, *MACROCYSTIS PYRIFERA*, GERMINATION AND GERM-TUBE LENGTH TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	34 ± 2‰
3. Temperature:	15 ± 1EC
4. Light quality:	Ambient laboratory illumination
5. Light intensity:	50 ± 10 µE/m^2/s
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Spore density per test chamber:	7500/mL of test solution
10. No. replicate chambers per concentration:	5
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine prepared from natural seawater
12. Test concentrations:	Effluents: Minimum of 5 and a control Receiving waters: 100% receiving water and a control
13. Dilution factor:	Effluents: $0.5 Receiving waters: None or $0.5
14. Test duration:	48 h

15. Endpoints:	Germination and germ-tube length
16. Test acceptability criteria:	$70% germination in the controls; $10 µm germ-tube length in the controls and the NOEC must be below 35 µg/L in the reference toxicant test; must achieve a %MSD of <20 for both germination and germ-tube length in the reference toxicant.
17. Sampling requirements:	One sample collected at test initiation, and preferably used within 24 h of the time it is removed from the sampling device (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests)
18. Sample volume required:	2 L per test

17.13 **DATA ANALYSIS**

17.13.1 GENERAL

17.13.1.1 Tabulate and summarize the data. Table 4 presents a sample set of germination and growth data.

17.13.1.2 The endpoints of the giant kelp 48-hour chronic test are based on the adverse effects on germination and growth. The IC25 endpoints are calculated using point estimation techniques (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for germination and growth 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 LOEC and NOEC endpoints and for the estimation of the IC25 endpoints. Concentrations at which there is no germination in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC for germination and growth, but included in the estimation of the

IC25. See the Appendices for examples of the manual computations, and examples of data input and program output.

17.13.1.3 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.

17.13.2 EXAMPLE OF ANALYSIS OF GIANT KELP, *MACROCYSTIS PYRIFERA*, GERMINATION DATA

17.13.2.1 Formal statistical analysis of the germination data is outlined in Figure 1. The response used in the analysis is the proportion of germinated spores in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 endpoint. Concentrations at which there is no germination in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC endpoints.

17.13.2.2 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, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, 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. 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 estimated by the parametric procedure.

17.13.2.3 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 (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

TABLE 4. DATA FROM GIANT KELP, *MACTOCYSTIS PYRIFERA* GERMINATION AND GROWTH TEST

Copper Conc. (µg/L)	Replicate Chamber	Number Counted	Number Germinated	Proportion Germinated	Mean Length
Control	1	100	89	0.89	19.58
	2	100	88	0.88	18.75
	3	100	85	0.85	19.14
	4	100	89	0.89	16.50
	5	100	91	0.91	17.93
5.6	1	100	82	0.82	18.26
	2	100	55	0.55	16.25
	3	100	84	0.84	16.39
	4	100	96	0.96	18.70
	5	100	85	0.85	15.62
10.0	1	100	90	0.90	13.31
	2	100	90	0.90	18.92
	3	100	70	0.70	15.62
	4	100	83	0.83	14.30
	5	100	87	0.87	15.29
18.0	1	100	88	0.88	18.59
	2	100	52	0.52	12.88
	3	100	83	0.83	16.28
	4	100	54	0.54	15.38
	5	100	49	0.49	19.75
32.0	1	100	71	0.71	12.54
	2	100	82	0.82	10.67
	3	100	86	0.86	15.95
	4	100	81	0.81	12.54
	5	100	82	0.82	11.66
56.0	1	100	84	0.84	11.44
	2	100	68	0.68	11.88
	3	100	62	0.62	11.88
	4	100	80	0.80	11.00
	5	100	83	0.83	11.55
100.0	1	100	66	0.66	7.92
	2	100	72	0.72	7.59
	3	100	63	0.63	8.25
	4	100	72	0.72	9.13
	5	100	71	0.71	8.80
180.0	1	100	37	0.37	6.49
	2	100	69	0.69	7.25
	3	100	0	0.00	--
	4	100	32	0.32	7.63
	5	100	48	0.48	8.13

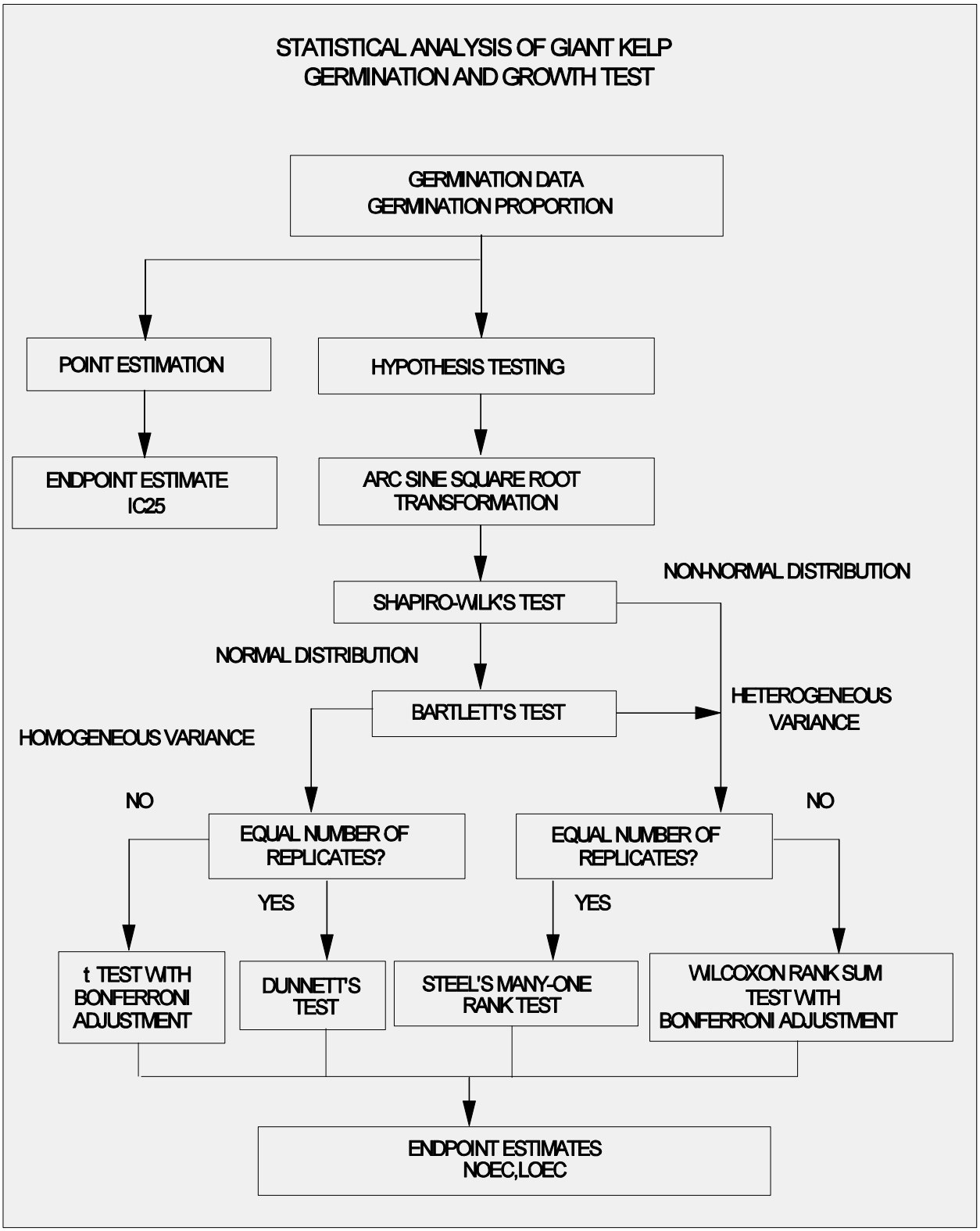

Figure 1. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, germination data.

17.13.2.4 Example of Analysis of Germination Data

17.12.2.4.1 This example used toxicity data from a giant kelp, Macrocystis pyrifera, germination and growth test performed with copper. The response of interest is the proportion of germinated spores, thus each replicate must be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each concentration including the control are listed in Table 5. A plot of the survival data is provided in Figure 2.

17.13.2.5 Test for Normality

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

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

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

Where: $\&_i$ = the ith centered observation

$\&$ = the overall mean of the centered observations

n = the total number of centered observations.

17.13.2.5.3 For this set of data, n = 40

$$\& = \frac{1}{40}(-0.002) = 0.000$$

D = 0.9281

17.13.2.5.4 Order the centered observations from smallest to largest:

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

TABLE 5. GIANT KELP, *MACTOCYSTIS PYRIFERA* GERMINATION DATA

COPPER CONCENTRATION (µg/L)	REPLICATE CHAMBER	RAW DATA	ARC SINE SQUARE ROOT TRANSFORMED	i	MEAN &	S_i^2
Control	1	0.89	1.233	1	1.224	0.00114
	2	0.88	1.217			
	3	0.85	1.173			
	4	0.89	1.233			
	5	0.91	1.266			
5.6	1	0.82	1.133	2	1.134	0.03670
	2	0.55	0.835			
	3	0.84	1.159			
	4	0.96	1.369			
	5	0.85	1.173			
10.0	1	0.90	1.249	3	1.167	0.01152
	2	0.90	1.249			
	3	0.70	0.991			
	4	0.83	1.146			
	5	0.87	1.202			
18.0	1	0.88	1.217	4	0.954	0.04423
	2	0.52	0.805			
	3	0.83	1.146			
	4	0.54	0.825			
	5	0.49	0.775			
32.0	1	0.71	1.002	5	1.115	0.00466
	2	0.82	1.133			
	3	0.86	1.187			
	4	0.81	1.120			
	5	0.82	1.133			
56.0	1	0.84	1.159	6	1.058	0.01272
	2	0.68	0.970			
	3	0.62	0.907			
	4	0.80	1.107			
	5	0.83	1.146			
100.0	1	0.66	0.948	7	0.979	0.00191
	2	0.72	1.013			
	3	0.63	0.917			
	4	0.72	1.013			
	5	0.71	1.002			
180.0	1	0.37	0.654	8	0.610	0.11914
	2	0.69	0.980			
	3	0.00	0.050			
	4	0.32	0.601			
	5	0.48	0.765			

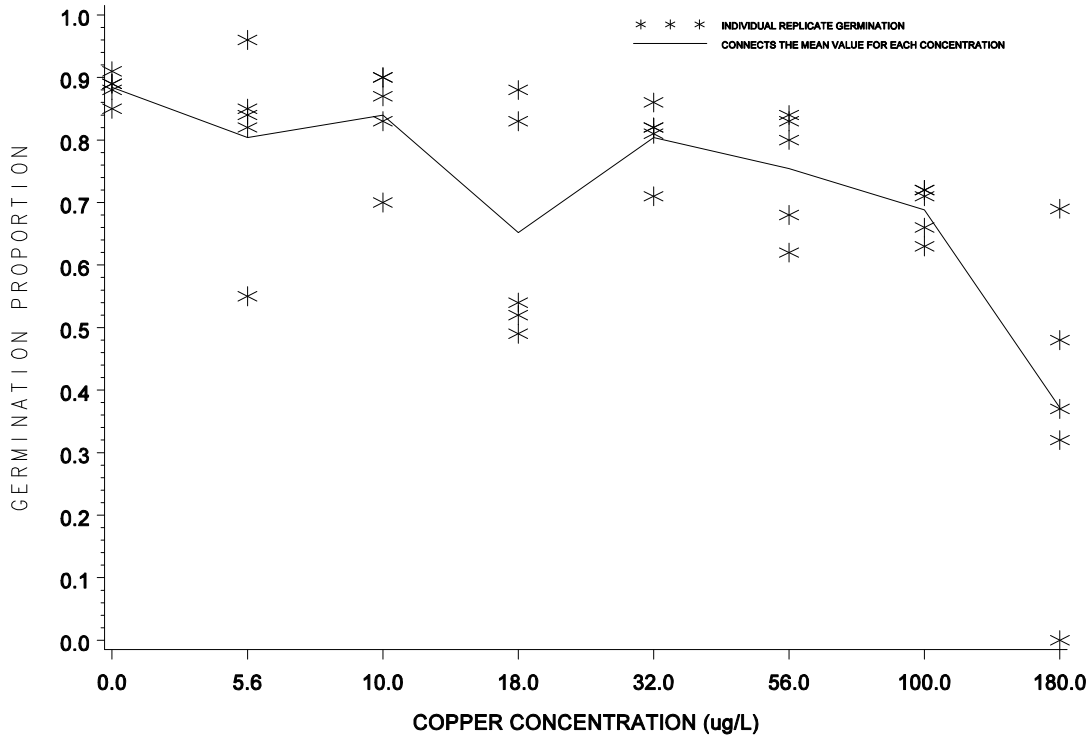

Figure 2. Plot of germination proportions of the giant kelp, *Macrocystis pyrifera*, at each treatment level.

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

	Copper Concentration (µg/L)							
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	0.009	-0.001	0.082	0.263	-0.113	0.101	-0.031	0.044
2	-0.007	-0.299	0.082	-0.149	0.018	-0.088	0.034	0.370
3	-0.051	0.025	-0.176	0.192	0.072	-0.151	-0.062	-0.560
4	0.009	0.235	-0.021	-0.129	0.005	0.049	0.034	-0.009
5	0.042	0.039	0.035	-0.179	0.018	0.088	0.023	0.155

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.560	21	0.018
2	-0.299	22	0.023
3	-0.179	23	0.025
4	-0.176	24	0.034
5	-0.151	25	0.034
6	-0.149	26	0.035
7	-0.129	27	0.039
8	-0.113	28	0.042
9	-0.088	29	0.044
10	-0.062	30	0.049
11	-0.051	31	0.072
12	-0.031	32	0.082
13	-0.021	33	0.082
14	-0.009	34	0.088
15	-0.007	35	0.101
16	-0.001	36	0.155
17	0.005	37	0.192
18	0.009	38	0.235
19	0.009	39	0.263
20	0.018	40	0.370

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

17.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2,...., 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 = 40 and k = 20. The a_i values are listed in Table 8.

17.13.2.5.6 Compute the test statistic, W, as follows:

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

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

$$W = \frac{1}{0.9281}(0.9230)^2 = 0.918$$

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3964	0.930	$X^{(40)} - X^{(1)}$
2	0.2737	0.562	$X^{(39)} - X^{(2)}$
3	0.2368	0.414	$X^{(38)} - X^{(3)}$
4	0.2098	0.368	$X^{(37)} - X^{(4)}$
5	0.1878	0.306	$X^{(36)} - X^{(5)}$
6	0.1691	0.250	$X^{(35)} - X^{(6)}$
7	0.1526	0.217	$X^{(34)} - X^{(7)}$
8	0.1376	0.195	$X^{(33)} - X^{(8)}$
9	0.1237	0.170	$X^{(32)} - X^{(9)}$
10	0.1108	0.134	$X^{(31)} - X^{(10)}$
11	0.0986	0.100	$X^{(30)} - X^{(11)}$
12	0.0870	0.075	$X^{(29)} - X^{(12)}$
13	0.0759	0.063	$X^{(28)} - X^{(13)}$
14	0.0651	0.048	$X^{(27)} - X^{(14)}$
15	0.0546	0.042	$X^{(26)} - X^{(15)}$
16	0.0444	0.035	$X^{(25)} - X^{(16)}$
17	0.0343	0.029	$X^{(24)} - X^{(17)}$
18	0.0244	0.016	$X^{(23)} - X^{(18)}$
19	0.0146	0.014	$X^{(22)} - X^{(19)}$
20	0.0049	0.000	$X^{(21)} - X^{(20)}$

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 5.6 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Germinated	Concentration
1	0.835	5.6 µg/L
2	1.133	5.6 µg/L
3	1.159	5.6 µg/L
4.5	1.173	5.6 µg/L
4.5	1.173	Control
6	1.217	Control
7.5	1.233	Control
7.5	1.233	Control
9	1.266	Control
10	1.369	5.6 µg/L

TABLE 10. TABLE OF RANKS[1]

Rep.	Control	Concentration (µg/L) 5.6	10.0
1	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.133(2)	1.249(8.5)
2	1.217(6,5,6.5,7,7,7,7)	0.835(1)	1.249(8.5)
3	1.173(4.5,3,5,5,6,6,6)	1.159(3)	0.991(1)
4	1.233(7.5,6.5,8.5,8.5,8.5,8.5,8.5)	1.369(10)	1.146(2)
5	1.266(9,10,10,10,10,10,10)	1.173(4.5)	1.202(4)

Concentration (µg/L) (Continued)

Rep.	18.0	32.0	56.0	100.0	180.0
1	1.217(6.5)	1.002(1)	1.159(5)	0.948(2)	0.654(3)
2	0.805(2)	1.133(3.5)	0.970(2)	1.013(4.5)	0.980(5)
3	1.146(4)	1.187(6)	0.907(1)	0.917(1)	0.050(1)
4	0.825(3)	1.120(2)	1.107(3)	1.013(4.5)	0.601(2)
5	0.775(1)	1.133(3.5)	1.146(4)	1.002(3)	0.765(4)

[1]Control ranks are given in the order of the concentration with which they were ranked.

17.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 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 set of data, the critical value at a significance level of 0.01 and n = 40 observations is 0.919. Since W = 0.918 is less than the critical value, conclude that the data are not normally distributed.

17.13.2.5.8 Since the data do not meet the assumption of normality, Steel's Many-one Rank Test will be used to analyze the germination data.

17.13.2.6 Steel's Many-one Rank Test

17.13.2.6.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ... , 10) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

17.13.2.6.2 An example of assigning ranks to the combined data for the control and 5.6 µg/L copper concentration is given in Table 9. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 10. The ranks are then summed for each concentration level, as shown in Table 11.

17.13.2.6.3 For this example, determine if the survival in any of the concentrations is significantly lower than the survival in the control. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus compare the rank sums for the survival at each of the various concentration levels with some "minimum" or critical rank sum, at or below which the survival would be considered significantly lower than the control. At a significance level of 0.05, the minimum rank sum in a test with seven concentrations (excluding the control) and five replicates is 16 (See Table 5, Appendix E).

17.13.2.6.4 Since the rank sum for the 32.0 µg/L concentration is equal to the critical value and the rank sums for the 56.0, 100.0 and 180.0 µg/L concentrations are less than the critical value, the germination proportions in those concentrations are considered significantly less than that in the control. Hence, the NOEC and the LOEC are considered to be 18.0 µg/L and 32.0 µg/L, respectively.

TABLE 11. RANK SUMS

Concentration	Rank Sum
5.6	20.5
10.0	24.0
18.0	16.5
32.0	16.0
56.0	15.0
100.0	15.0
180.0	15.0

17.13.2.7 Calculation of the ICp

17.13.2.7.1 The germination data from Table 4 and Figure 2 are utilized in this example. As can be seen from the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC.

17.13.2.7.2 Starting with the observed control mean, $\overline{Y}_1 = 0.884$ is less than the observed mean for the lowest effluent concentration, $\overline{Y}_2 = 0.804$, so set $M_1 = 0.884$.

17.13.2.7.3 Comparing $\overline{Y}_2$ to $\overline{Y}_3 = 0.840$, we see that $\overline{Y}_2$ is less than $\overline{Y}_3$.

17.13.2.7.4 Calculate the smoothed means:

$$M_2 = M_3 = (\overline{Y}_2 + \overline{Y}_3)/2 = 0.822$$

17.13.2.7.5 Since M_3 is larger than $\bar{Y}_4 = 0.652$, set $M_4 = 0.652$. Since $\bar{Y}_5 = 0.804$ is larger than M_4, these means must be smoothed.

17.13.2.7.6 Calculate the smoothed means:

$$M_4 = M_5 = (M_4 + \bar{Y}_5)/2 = 0.728.$$

17.13.2.7.7 Since $\bar{Y}_6 = 0.754$ is larger than M_5, average $\bar{Y}_6$ with the two previous concentrations:

$$M_4 = M_5 = M_6 = (M_4 + M_5 + \bar{Y}_6)/3 = 0.737.$$

17.13.2.7.8 Since $M_6 > \bar{Y}_7 = 0.688 > \bar{Y}_8 = 0.372$, set $M_7 = 0.688$ and $M_8 = 0.372$. Table 12 contains the smoothed means and Figure 3 gives a plot of the smoothed means and the interpolated response curve.

17.13.2.7.9 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in germination, compared to the controls, would result in a mean germination of 0.663, where $M_1(1-p/100) = 0.884(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 12), the response, 0.663, is bracketed by $C_7 = 100.0$ µg/L and $C_8 = 180.0$ µg/L.

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

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 100.0 + [0.884(1 - 25/100) - 0.688] (180.0 - 100.0) S))))))))))))))Q (0.372 - 0.688)$$

$$= 106.3 \text{ µg/L}.$$

17.13.2.7.11 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 106.3291 µg/L. The empirical 95.0% confidence interval for the true mean was 94.6667 µg/L to 117.0588 µg/L. The computer program output for the IC25 for this data set is shown in Figure 4.

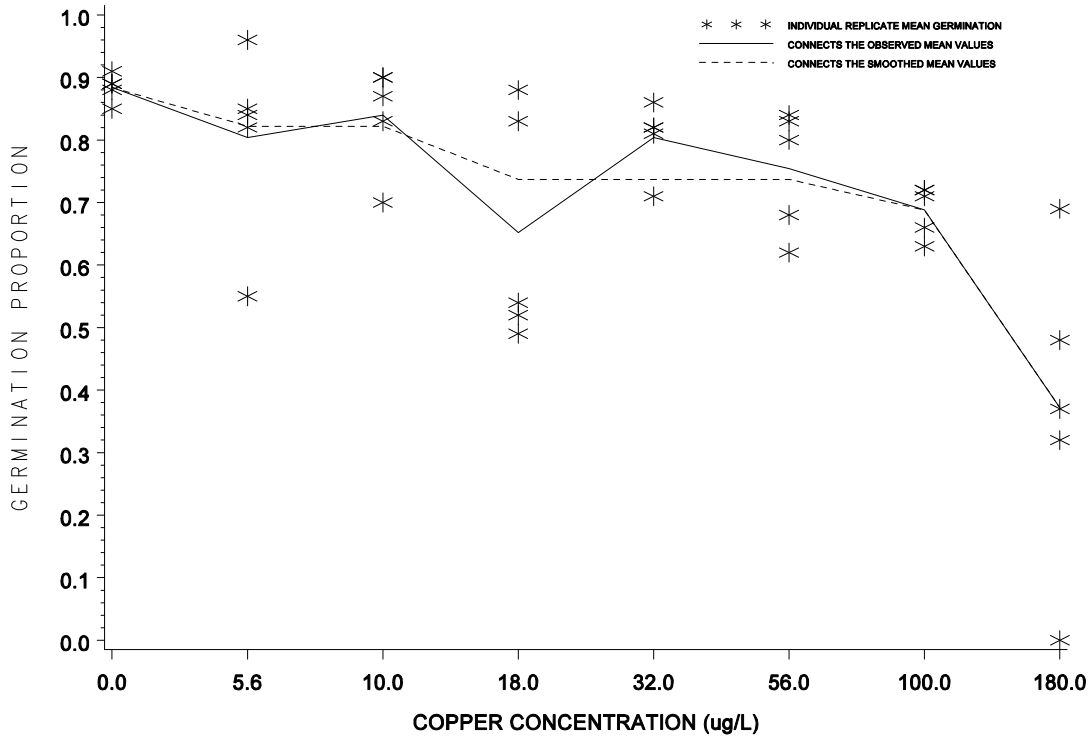

Figure 3. Plot of raw data, observed means, and smoothed means for the giant kelp, *Macrocystis pyrifera*, germination data from Tables 4 and 13.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  5.6   10   18   32   56  100  180
-------------------------------------------------------
Response  1   .89  .82  .90  .88  .71  .84  .66  .37
Response  2   .88  .55  .90  .52  .82  .68  .72  .69
Response  3   .85  .84  .70  .83  .86  .62  .63    0
Response  4   .89  .96  .83  .54  .81  .80  .72  .32
Response  5   .91  .85  .87  .49  .82  .83  .71  .48
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Giant Kelp, Macrocystis pyrifera
Test Duration:        48 hours
DATA FILE: kelpgerm.icp
OUTPUT FILE: kelpgerm.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates          ug/L        Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          5            0.000         0.884      0.022      0.884
  2          5            5.600         0.804      0.152      0.822
  3          5           10.000         0.840      0.083      0.822
  4          5           18.000         0.652      0.187      0.737
  5          5           32.000         0.804      0.056      0.737
  6          5           56.000         0.754      0.098      0.737
  7          5          100.000         0.688      0.041      0.688
  8          5          180.000         0.372      0.252      0.372
-------------------------------------------------------------------------
The Linear Interpolation Estimate:   106.3291   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean: 105.8680 Standard Deviation:     5.6981
Original Confidence Limits:   Lower:    94.6667 Upper:   117.0588
Expanded Confidence Limits:   Lower:    88.8354 Upper:   122.4237
Resampling time in Seconds:     0.28  Random_Seed: 390692880
```

Figure 4. ICPIN program output for the IC25.

17.13.3 EXAMPLE OF ANALYSIS OF GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

17.13.3.1 Formal statistical analysis of the growth data is outlined in Figure 5. The response used in the statistical analysis is mean germ-tube length per replicate. An IC25 can be calculated for the growth data via a point estimation technique (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). Hypothesis testing can be used to obtain the NOEC and LOEC for growth.

17.13.3.2 The statistical analysis using hypothesis tests consists of a parametric test, Dunnett's Procedure, and a nonparametric test, Steel's Many-one Rank Test. The underlying assumptions of the Dunnett's Procedure, 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. 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.

17.13.3.3 Additionally, 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. The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative. For detailed information on the Bonferroni adjustment, see Appendix D.

17.13.3.4 The data, mean and variance of the observations at each concentration including the control for this example are listed in Table 13. A plot of the data is provided in Figure 6.

17.13.3.5 Test for Normality

17.13.3.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are listed in Table 14.

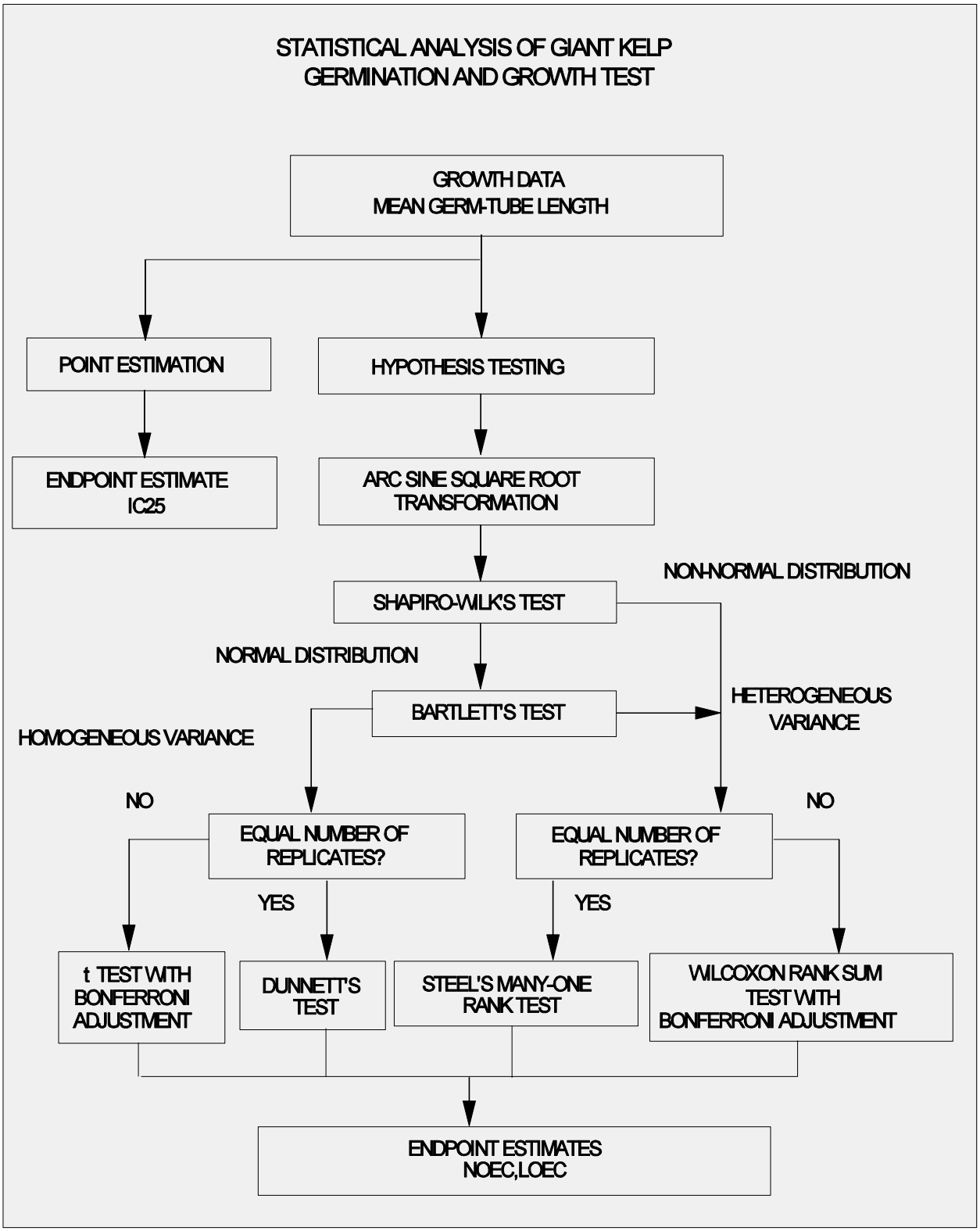

Figure 5. Flowchart for statistical analysis of giant kelp, *Macrocystis pyrifera*, growth data.

TABLE 13. GIANT KELP, *MACROCYSTIS PYRIFERA*, GROWTH DATA

	Copper Concentration (µg/L)							
Rep	Control	5.60	10.0	18.0	32.0	56.0	100.0	180.0
1	19.58	18.26	13.31	18.59	12.54	11.44	7.92	6.49
2	18.75	16.25	18.92	12.88	10.67	11.88	7.59	7.25
3	19.14	16.39	15.62	16.28	15.95	11.88	8.25	--
4	16.50	18.70	14.30	15.38	12.54	11.00	9.13	7.63
5	17.93	15.62	15.29	19.75	11.66	11.55	8.80	8.13
Mean($\bar{X}_i$)	18.38	17.04	15.49	16.58	12.67	11.55	8.34	7.38
S_i^2	1.473	1.827	4.498	7.327	3.953	0.133	0.396	0.478
i	1	2	3	4	5	6	7	8

TABLE 14. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Copper Concentration (µg/L)							
Rep	Control	5.6	10.0	18.0	32.0	56.0	100.0	180.0
1	1.20	1.22	-2.18	2.01	-0.13	-0.11	-0.42	-0.89
2	0.37	-0.79	3.43	-3.70	-2.00	0.33	-0.75	-0.13
3	0.76	-0.65	0.13	-0.30	3.28	0.33	-0.09	--
4	-1.88	1.66	-1.19	-1.20	-0.13	-0.55	0.79	0.25
5	-0.45	-1.42	-0.20	3.17	-1.01	0.00	0.46	0.75

17.13.3.5.2 Calculate the denominator, D, of the statistic:

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

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

17.13.3.5.3 For this set of data, n = 39

$$\bar{X} = \frac{1}{39}(-0.03) = 0.000$$

D = 79.8591

17.13.3.5.4 Order the centered observations from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 15.

TABLE 15. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-3.70	21	-0.11
2	-2.18	22	-0.09
3	-2.00	23	0.00
4	-1.88	24	0.13
5	-1.42	25	0.25
6	-1.20	26	0.33
7	-1.19	27	0.33
8	-1.01	28	0.37
9	-0.89	29	0.46
10	-0.79	30	0.75
11	-0.75	31	0.76
12	-0.65	32	0.79
13	-0.55	33	1.20
14	-0.45	34	1.22
15	-0.42	35	1.66
16	-0.30	36	2.01
17	-0.20	37	3.17
18	-0.13	38	3.28
19	-0.13	39	3.43
20	-0.13		

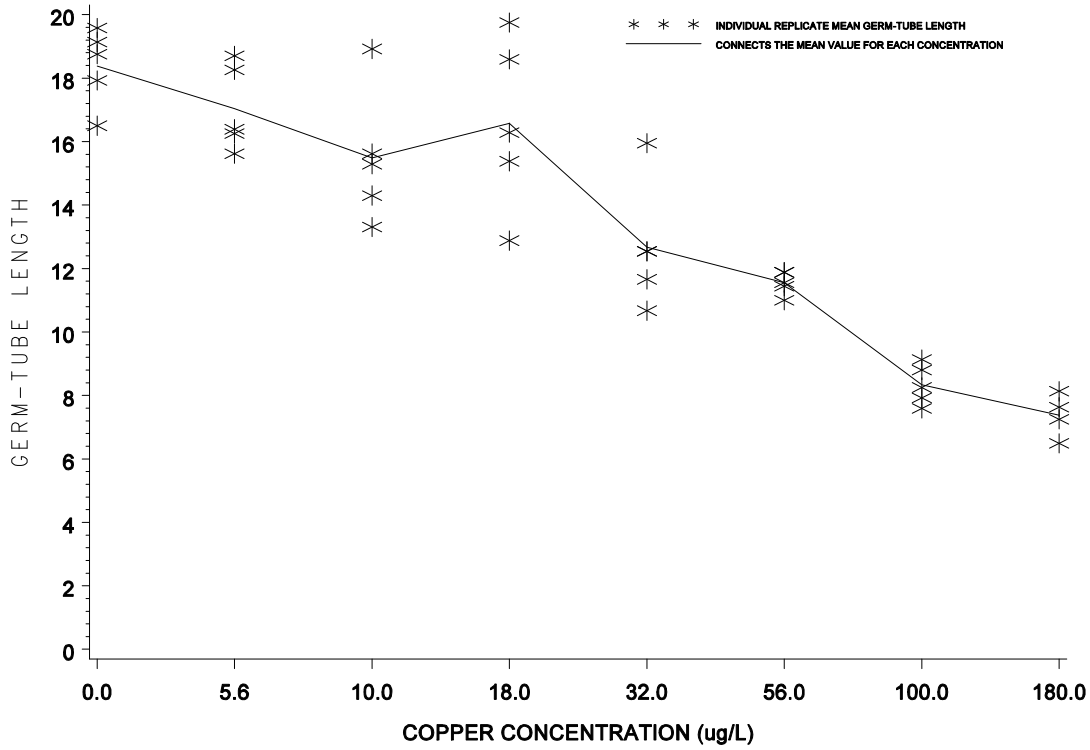

Figure 6. Plot of mean growth data for the giant kelp, *Macrocystis pyrifera*, tests.

17.13.3.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients a_1, a_2, ... 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 = 39 and k = 19. The a_i values are listed in Table 16.

17.13.3.5.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 16. For this set of data:

$$W = \frac{1}{79.8591}(8.7403)^2 = 0.957$$

TABLE 16. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.3989	7.13	$X^{(39)} - X^{(1)}$
2	0.2755	5.46	$X^{(38)} - X^{(2)}$
3	0.2380	5.17	$X^{(37)} - X^{(3)}$
4	0.2104	3.89	$X^{(36)} - X^{(4)}$
5	0.1880	3.08	$X^{(35)} - X^{(5)}$
6	0.1689	2.42	$X^{(34)} - X^{(6)}$
7	0.1520	2.39	$X^{(33)} - X^{(7)}$
8	0.1366	1.80	$X^{(32)} - X^{(8)}$
9	0.1225	1.65	$X^{(31)} - X^{(9)}$
10	0.1092	1.54	$X^{(30)} - X^{(10)}$
11	0.0967	1.21	$X^{(29)} - X^{(11)}$
12	0.0848	1.02	$X^{(28)} - X^{(12)}$
13	0.0733	0.88	$X^{(27)} - X^{(13)}$
14	0.0622	0.78	$X^{(26)} - X^{(14)}$
15	0.0515	0.67	$X^{(25)} - X^{(15)}$
16	0.0409	0.43	$X^{(24)} - X^{(16)}$
17	0.0305	0.20	$X^{(23)} - X^{(17)}$
18	0.0203	0.04	$X^{(22)} - X^{(18)}$
19	0.0101	0.02	$X^{(21)} - X^{(19)}$

17.13.3.5.7 The decision rule for this test is to compare W as calculated in Subsection 5.6 to a 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 set of data, the critical value at a significance level of 0.01 and n = 39 observations is 0.917. Since W = 0.957 is greater than the critical value, conclude that the data are normally distributed.

17.13.3.6 Test for Homogeneity of Variance

17.13.3.6.1 The test used to examine whether the variation in mean weight of the mysids is the same across all concentration levels including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{P} V_i)\ 1n\ \bar{S}^2 - \sum_{i=1}^{P} V_i\ 1n\ S_i^2]}{C}$$

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

p = number of concentration levels including the control

ln = $\log_e$

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

n_i = the number of replicates for concentration i.

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

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

17.13.3.6.2 For the data in this example (See Table 13), all concentrations including the control have five replicates except the 180 µg/L concentration which has four replicates (n_i = 5 for i = 1 - 7; n_8 = 4). Thus, V_i = 4 for i = 1 - 7 and V_8 = 3.

17.13.3.6.3 Bartlett's statistic is therefore:

$$B = [(31)\ln(2.5761) - \sum_{i=1}^{P} V_i \ln(S_i^2)] / 1.0977$$

= [31(0.9463) - [4ln(1.4729) + ... + 3ln(0.4780)] / 1.0977

= [29.3353 - 9.4481] / 1.0977

= 18.12

17.13.3.6.4 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 seven degrees of freedom, is 18.48. Since B = 18.12 is less than the critical value, conclude that the variances are not different.

17.13.3.7 *t* Test with Bonferroni's Adjustment

17.13.3.7.1 To obtain an estimate of the pooled variance for the *t* test with Bonferroni's adjustment, construct an ANOVA table as described in Table 17.

TABLE 17. 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 of concentration levels including the control

N = total number of observations $n_1 + n_2 \ldots + n_p$

n_i = number of observations in concentration i

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

$$SST = \sum_{i=1}^{P} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$ Total Sum of Squares

$SSW = SST - SSB$ 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 length of the germ-tubes for concentration i in test chamber j)

17.13.3.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = n_7 = 5;\ n_8 = 4$

$N = 39$

$T_1 = Y_{11} + Y_{12} + Y_{13} + Y_{14} + Y_{15} = 91.90$
$T_2 = Y_{21} + Y_{22} + Y_{23} + Y_{24} + Y_{25} = 85.22$
$T_3 = Y_{31} + Y_{32} + Y_{33} + Y_{34} + Y_{35} = 77.44$
$T_4 = Y_{41} + Y_{42} + Y_{43} + Y_{44} + Y_{45} = 82.88$
$T_5 = Y_{51} + Y_{52} + Y_{53} + Y_{54} + Y_{55} = 63.36$
$T_6 = Y_{61} + Y_{62} + Y_{63} + Y_{64} + Y_{65} = 57.75$
$T_7 = Y_{71} + Y_{72} + Y_{73} + Y_{74} + Y_{75} = 41.69$
$T_8 = Y_{81} + Y_{82} + Y_{83} + Y_{84} = 29.50$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 + T_7 + T_8 = 529.74$

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

$$= 7749.905 - \frac{(529.74)^2}{39} = 554.406$$

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

$$= 7829.764 - \frac{(529.74)^2}{39} = 634.265$$

$$SSW = SST - SSB = 634.265 - 554.406 = 79.859$$

S_B^2 = SSB/(p-1) = 554.406/(8-1) = 79.201

S_W^2 = SSW/(N-p) = 79.859/(39-8) = 2.576

17.13.3.7.3 Summarize these calculations in the ANOVA table (Table 18).

TABLE 18. ANOVA TABLE FOR THE *t* TEST WITH BONFERRONI'S ADJUSTMENT EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	7	554.406	79.201
Within	31	79.859	2.576
Total	38	634.265	

17.13.3.7.4 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: $\bar{x}_i$ = mean length for concentration i

$\bar{x}_1$ = mean length 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

17.13.3.7.5 Table 19 includes the calculated *t* values for each concentration and control combination. In this example, comparing the 5.6 µg/L concentration with the control, the calculation is as follows:

$$t_2 = \frac{(18.38 - 17.04)}{[1.605\sqrt{(1/5)+(1/5)}]}$$

$$= 1.320$$

TABLE 19. CALCULATED *t* VALUES

Concentration (µg/L)	i	t_i
5.6	2	1.320
10.0	3	2.847
18.0	4	1.773
32.0	5	5.625
56.0	6	6.728
100.0	7	9.891
180.0	8	10.836

17.13.3.7.6 Since the purpose of this test is to detect a significant reduction in mean length, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D. For an overall alpha level of 0.05, 31 degrees of freedom for error and seven concentrations (excluding the control) the approximate critical value is 2.597. The mean weight for concentration "i" is considered significantly less than the mean weight for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L, 32 µg/L, 56.0 µg/L,

100.0 µg/L, 180.0 µg/L concentrations have significantly lower mean length than the control. Because the 10.0 µg/L concentration shows signigicantly lower mean length than the control while the higher 18.0 µg/L concentration does not, these test results are considered to have an anomalous dose-response relationship and it is recommended that the test be repeated. If an NOEC and LOEC must be determined for this test, the lowest concentration with significant growth impairment versus the control is considered to the LOEC for growth. Thus, for this test, the NOEC and LOEC would be 5.6 µg/L and 10.0 µg/L, respectively.

17.13.3.8 Calculation of the ICp

17.13.3.8.1 The growth data from Table 13 and Figure 3 are utilized in this example. As can be seen in the figure, the observed means are not monotonically non-increasing with respect to concentration. Therefore, the means must be smoothed prior to calculating the IC

17.13.3.8.2 Starting with the observed control mean, $\overline{Y}_1 = 18.38$ is greater than the observed mean for the lowest copper concentration, $\overline{Y}_2 = 17.044$, so set $M_1 = 18.38$. Likewise, $\overline{Y}_2$ is greater than the observed mean for the next copper concentration, $\overline{Y}_3 = 15.488$, so set $M_2 = 17.044$.

17.13.3.8.3 Comparing $\overline{Y}_3$ to $\overline{Y}_4 = 16.576$, we see that $\overline{Y}_3$ is less than $\overline{Y}_4$.

17.13.3.8.4 Calculate the smoothed means:

$$M_3 = M_4 = (\overline{Y}_3 + \overline{Y}_4)/2 = 16.032$$

17.13.3.8.5 Since $M_4 > \overline{Y}_5 = 12.672 > \overline{Y}_6 = 11.550 > \overline{Y}_7 = 8.338 > \overline{Y}_8 = 7.375$, set $M_5 = 12.672$, $M_6 = 11.550$, $M_7 = 8.338$ and $M_8 = 7.375$. Table 20 contains the smoothed means and Figure 7 gives a plot of the smoothed response curve.

TABLE 20. GIANT KELP, *MACROCYSTIS PYRIFERA*, MEAN GERM-TUBE LENGTHS AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means $\bar{x}_i$ (mm)	Smoothed Means M_i (mm)
Control	1	18.380	18.380
5.6	2	17.044	17.044
10.0	3	15.488	16.032
18.0	4	16.576	16.032
32.0	5	12.672	12.672
56.0	6	11.550	11.550
100.0	7	8.338	8.338
180.0	8	7.375	7.375

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

$$ICp \text{ ' } C_j\%[M_1(1\&p/100)\&M_j]\frac{(C_{(j\%1)}\&C_j)}{(M_{(j\%1)}\&M_j)}$$

$$IC25 = 18.0 + [18.380(1 - 25/100) - 16.032] \frac{(32.0 - 18.0)}{(12.672 - 16.032)}$$

$$= 27.36 \text{ µg/L.}$$

17.13.3.8.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in length, compared to the controls, would result in a mean length of 13.785 mm, where $M_1(1-p/100) = 18.380(1-25/100)$. Examining the smoothed means and their associated concentrations (Table 20), the response, 13.785 mm, is bracketed by C_4 = 18.0 µg/L and C_5 = 32.0 µg/L.

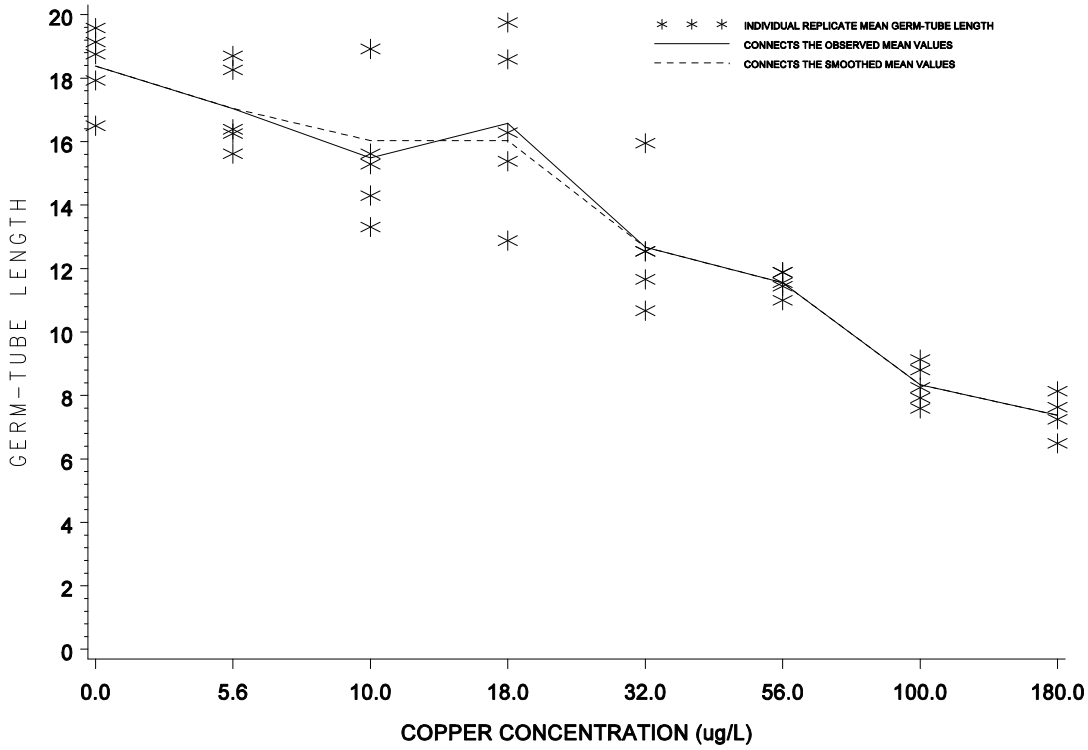

Figure 7. Plot of raw data, observed means, and smoothed means for the giant Kelp, *Macrocystis pyrifera*, growth data from Tables 13 and 20.

17.13.3.8.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 27.3625 µg/L. The empirical 95.0% confidence interval for the true mean was 20.8734 µg/L to 42.3270 µg/L. The computer program output for the IC25 for this data set is shown in Figure 8.

17.14 **PRECISION AND ACCURACY**

17.14.1 PRECISION

17.14.1.1 Single-Laboratory Precision

17.14.1.1.1 Single-laboratory precision data for the giant kelp 48-hour test method with the reference toxicants copper chloride and sodium azide with natural seawater are provided in Tables 21-22. The coefficient of variation (CV) of the germination EC50s using copper was 38.8%; the CV of the germ-tube length IC40s using copper was 32.9% (Table 21). The coefficient of variation (CV) of the germination EC50s using azide was 36.7%; the CV of the germ-tube length IC25s using azide was 30.8%, the CV of the germ-tube length IC50s using azide was 28.4% (Table 22).

17.14.1.2 Multi-laboratory Precision

17.14.1.2.1 Multi-laboratory precision data for the kelp 48-hour test method with the reference toxicant copper chloride are provided in Table 23. The coefficient of variation of the IC50s for the germ-tube length endpoint ranged between 8.4% and 55.5% using copper chloride. The coefficient of variation of the IC50s for the germination endpoint ranged between >1.1% and 67.6% using copper chloride.

17.14.2 ACCURACY

17.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID        1    2    3    4    5    6    7    8
-------------------------------------------------------
Conc. Tested    0  5.6   10   18   32   56  100  180
-------------------------------------------------------
Response  1 19.5818.2613.3118.5912.5411.44 7.92 6.49
Response  2 18.7516.2518.9212.8810.6711.88 7.59 7.25
Response  3 19.1416.3915.6216.2815.9511.88 8.25
Response  4 16.5018.7014.3015.3812.5411.00 9.13 7.63
Response  5 17.9315.6215.2919.7511.6611.55 8.80
-------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: Giant kelp, Macrocystis pyrifera
Test Duration:        48 hours
DATA FILE: kelpgrow.icp
OUTPUT FILE: kelpgrow.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          5             0.000         18.380      1.214     18.380
  2          5             5.600         17.044      1.352     17.044
  3          5            10.000         15.488      2.121     16.032
  4          5            18.000         16.576      2.707     16.032
  5          5            32.000         12.672      1.988     12.672
  6          5            56.000         11.550      0.365     11.550
  7          5           100.000          8.338      0.629      8.338
  8          4           180.000          7.375      0.691      7.375
-------------------------------------------------------------------------
The Linear Interpolation Estimate:    27.3625   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:  27.5292 Standard Deviation:     4.7812
Original Confidence Limits:   Lower:    20.8734 Upper:    42.3270
Expanded Confidence Limits:   Lower:    17.6289 Upper:    49.8093
Resampling time in Seconds:     0.28  Random_Seed: -35158431
```

Figure 8. ICPIN program output for the IC25.

TABLE 21. SINGLE LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST WITH COPPER (CU FG/L) CHLORIDE AS THE REFERENCE TOXICANT

Test Number	Germ-Tube Length		Germination	
	NOEC	IC40	NOEC	EC50
1	<5.6	122.7	10.0	67.5
2	10.0	43.1	18.0	73.5
3	18.0	70.7	18.0	124.3
4	5.6	88.0	56.0	101.6
5	32.0	124.7	56.0	122.9
Mean	89.8		90.7	
CV	38.8%		32.9%	

Data from Anderson et al., 1994

TABLE 22. SINGLE LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST WITH SODIUM AZIDE (MG/L) AS THE REFERENCE TOXICANT

Test Date	Germ-Tube Length			Germination	
	NOEC	IC25	IC50	NOEC	EC50
2/11/92	18.0	39.5	133.7	18.0	52.3
2/18/92	18.0	34.1	96.5	32.0	72.6
6/29/92	32.0	57.5	142.2	32.0	132.1
7/07/92	10.0	33.1	92.5	10.0	79.2
7/15/92	18.0	42.8	138.9	18.0	117.8
7/16/92	5.6	25.0	68.4	10.0	48.3
7/22/92	10.0	30.2	80.6	18.0	62.4
10/09/92	5.6	25.1	80.0	5.6	60.3
7/02/92	10.0	24.8	80.1	18.0	84.0
Mean	34.7		101.4	78.8	
CV	30.8%		28.4%	36.7%	

Data from Hunt et al., 1991

TABLE 23. MULTI-LABORATORY PRECISION OF THE GIANT KELP, *MACROCYSTIS PYRIFERA* GERMINATION AND GERM-TUBE LENGTH TEST PERFORMED WITH COPPER CHLORIDE (µG/L) AS THE REFERENCE TOXICANT

	Lab	Germ-tube length NOEC	IC40	Germination NOEC	EC50	CV Germ-tube	CV Germination
March 1990	1	5.6	122.7	10.0	46.9	8.4%	>1.1%
	2	32.0	117.8	32.0	46.2		
	3	18.0	104.1	32.0	*		
May 1990	1	10.0	43.1	18.0	112.0	39.9%	59.3%
	2	<5.6	99.1	32.0	164.2		
	3	18.0	68.7	18.0	67.9		
May 1990	1	18.0	70.7	18.0	112.0	45.3%	nc
	2	18.0	91.3	56.0	64.5		
	3	32.0	134.2	32.0	158.0		
December 1990	1	5.6	88.0	56.0	77.7	45.3%	nc
	2	5.6	45.3	18.0	*		
September 1990	1	32.0	124.7	56.0	127.4	55.5%	7.4%
	2	18.0	54.4	56.0	114.8		
September 1989	1	<10.0	89.3**	56.0	115.5	44.5%	67.6%
	2	<10.0	171.8**	56.0	327.7		
November 1989	1	32.0	>180.0	<10.0	>180.0	nc	nc
	2	10.0	>180.0	18.0	>180.0		
May 1988	1	<56.0	232.0***	<56.0	211.0	nc	50.0%
	2	<56.0	*	56.0	100.7		

* No EC50 calculated because response was less than 50%.
** Only concentration means available, therefore no IC40 values were calculated.
nc Not calculated (Insufficient numbers to calculate the coefficient of variation).
*** IC50 value, not IC40

Data from Hunt et al., 1991

APPENDIX I. MACROCYSTIS TEST: STEP-BY-STEP SUMMARY

PREPARATION OF TEST SOLUTIONS

A. Determine test concentrations and appropriate dilution water based on NPDES permit conditions and guidance from the appropriate regulatory agency.

B. Prepare effluent test solutions by diluting well mixed unfiltered effluent using volumetric flasks and pipettes. Use hypersaline brine where necessary to maintain all test solutions at 34 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution (10,000 µg/L) by adding 0.0268 of copper chloride ($CuCl_2 \circ 2H_2O$) to 1 liter of reagent water.

D. Prepare copper reference toxicant solution of 0 (control) 5.6, 10, 18, 32, 100 and 180 µg/L by adding 0, 0.56, 1.0 1.8, 3.2, 10.0 and 18.0 mL of stock solution, respectively, to a 1-L volumetric flask and filling to 1-L with dilution water.

E. Sample effluent and reference toxicant solutions for physical/chemical analysis. Measure salinity, pH and dissolved oxygen from each test concentration.

F. Randomize numbers for test chambers and record the chamber numbers with their respective test concentrations on a randomization data sheet. Store the data sheet safely until after the test samples have been analyzed.

G. Place test chambers in a water bath or environmental chamber set to 15EC and allow temperature to equilibrate.

H. Measure the temperature daily in one random replicate (or separate chamber) of each test concentration. Monitor the temperature of the water bath or environmental chamber continuously.

I. At the end of the test, measure salinity, pH, and dissolved oxygen concentration from each test concentration.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Collect sporophylls and rinse in 0.2 µm filtered seawater. Store at 9-12EC for no more than 24 hours before zoospore release.

B. Blot sporophylls and leave exposed to air for one hour.

C. Place 25-30 sporophylls one liter of 0.2 µm filtered seawater for no more than two hours. The presence of zoospores is indicated by a slight cloudiness in the water.

D. Take a sample of the zoospore solution from the top 5 centimeters of the beaker and determine the spore density using a hemacytometer. Determine the volume of water necessary to give 7,500 spores/mL of test solution. This volume should not exceed one percent of the test solution volume.

E. Verify that the zoospores are swimming, then pipet the volume of water necessary to give 7,500 spores/mL into each of the test chambers. Take zoospores from the top 5 centimeters of the release beaker so that only swimming zoospores are used.

F. At 48 ± 3 hours, count the number of germinated and non-germinated spores of the first 100 spores encountered in each replicate of each concentration. Measure the length of 10 randomly selected germination tubes (or preserve with 0.1% glutaraldehyde for later examination).

G. Analyze the data.

H. Include standard reference toxicant point estimate values in the standard quality control charts.

Data Sheet for Kelp Toxicity Test

Test Start Date: Start Time: Kelp Species :
Test End Date: End Time: Collection/Arrival Date:
Reference Toxicant: Kelp Source:

Sample Source: Microscope Model:
Sample Type: Solid Elutriate Pore Water Water Effluent Ref Tox

Micrometer Conversion Factor:

Test Cont. #	Station Code	Number of Spores Germ.	Number of Spores Not Germ.	Length Measurements (in ocular micrometer units)										Notes
				L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
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Computer Data Storage
Disk:
File: