SECTION 13

PACIFIC OYSTER, *Crassostrea gigas* AND MUSSEL, *Mytilus sp.* EMBRYO-LARVAL DEVELOPMENT TEST METHOD

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PACIFIC OYSTER, *CRASSOSTREA GIGAS*, AND MUSSEL, *MYTILUS SPP.* EMBRYO-LARVAL DEVELOPMENT TEST

13.1 SCOPE AND APPLICATION

13.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the embryos and larvae of several bivalve molluscs, the Pacific oyster (*Crassostrea gigas*) and the mussels (*Mytilus edulis*, *M. californianus*, *M. galloprovincialis*, or *M. trossulus*) in 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.

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

13.1.3 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.

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

13.1.5 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.

13.2 SUMMARY OF METHOD

13.2.1 The method provides step-by-step instructions for performing a 48-h static non-renewal toxicity test using embryos and larvae of the test species to determine the toxicity of

substances in marine and estuarine waters. The test endpoint is normal shell development and should include mortality as a measure of adverse effect.

13.3 **INTERFERENCES**

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

13.3.2 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).

13.4 **SAFETY**

13.4.1 See Section 3, Health and Safety

13.5 **APPARATUS AND EQUIPMENT**

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

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

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

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

13.5.5 Refractometer -- for determining salinity.

13.5.6 Hydrometer(s) -- for calibrating refractometer.

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

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

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

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

13.5.11 Winkler bottles -- for dissolved oxygen determinations.

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

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

13.5.14 Glass stirring rods -- for mixing test solutions.

13.5.15 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).

13.5.16 Volumetric flasks -- Class A, borosilicate glass or non-toxic plastic labware, 100-1000 mL for making test solutions.

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

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

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

13.5.20 Wash bottles -- for dilution water.

13.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

13.5.22 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.

13.5.23 Beakers, 50 mL -- for pooling surrogate water samples for chemistry measurements at the end of the test.

13.5.24 Beakers, 250 mL borosilicate glass -- for preparation of test solutions.

13.5.25 Beakers, 1,000 mL borosilicate glass -- for mixing gametes for fertilization of eggs.

13.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of embryos and larvae. The use of an inverted scope is not required, but recommended. Its use reduces the exposure of workers to hazardous fumes (formalin or glutaraldehyde) during the counting of larvae and reduces sample examination time. Alternatively, a Sedgewick-Rafter cell may be used on a regular compound scope.

13.5.27 Counter, two unit, 0-999 -- for recording counts of embryos and larvae.

13.5.28 A perforated plunger -- for maintaining a homogeneous suspension of embryos.

13.5.29 Nytex screens, ca. 75 µm and ca. 37 µm -- for rinsing gametes to separate individual gametes from larger material; for retaining eggs, embryos, or larvae.

13.5.30 60 µm NITEX® filter -- for filtering receiving water.

13.6 **REAGENTS AND SUPPLIES**

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

13.6.2 Data sheets (one set per test) -- for data recording (see Figure 1).

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

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

13.6.5 Parafilm -- to cover graduated cylinders and vessels containing gametes, embryos.

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

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

13.6.8 Pipet tips -- for automatic pipets.

13.6.9 Coverslips -- for microscope slides.

13.6.10 Lens paper -- for cleaning microscope optics.

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

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

13.6.13 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).

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

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

13.6.16 Test chambers -- 30 mL, four chambers per concentration. The chambers should be borosilicate glass or nontoxic disposable

plastic labware. The test may be performed in other sized chambers as long as the density of embryos is the same.

13.6.17 Formaldehyde, 37% (Concentrated Formalin) -- for preserving larvae. Note: formaldehyde has been identified as a carcinogen and is irritating to skin and mucous membranes. It should not be used at a concentration higher than necessary to achieve morphological preservation of larvae for counting and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.6.19 Reference toxicant solutions (see Section 13.10.2.4 and Section 4, Quality Assurance).

13.6.20 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).

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

13.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 13.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

13.6.23 HYPERSALINE BRINES

13.6.23.1 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.

13.6.23.2 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).

13.6.23.3 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.

13.6.23.4 Freeze Preparation of Brine

13.6.23.4.1 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).

13.6.23.4.2 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%.

13.6.23.4.3 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

TABLE 1. MAXIMUM EFFLUENT CONCENTRATION (%) THAT CAN BE TESTED BY ADDING DILUTION WATER ONLY OR BRINE ONLY (WITHOUT ADDITION OF DRY SEA SALTS), GIVEN VARIOUS EFFLUENT SALINITIES, DILUTION WATER SALINITIES, AND BRINE SALINITIES, AND MAINTAINING 30‰ TEST SALINITY.

Effl.	Dilution Water Salinity ‰					Brine Salinity ‰				
‰	31	32	33	34	35	60	70	80	90	100
0	3.23	6.25	9.09	11.76	14.29	50.00	57.14	62.50	66.67	70.00
1	3.33	6.45	9.38	12.12	14.71	50.85	57.97	63.29	67.42	70.71
2	3.45	6.67	9.68	12.50	15.15	51.72	58.82	64.10	68.18	71.43
3	3.57	6.90	10.00	12.90	15.63	52.63	59.70	64.94	68.97	72.16
4	3.70	7.14	10.34	13.33	16.13	53.57	60.61	65.79	69.77	72.92
5	3.85	7.41	10.71	13.79	16.67	54.55	61.54	66.67	70.59	73.68
10	4.76	9.09	13.04	16.67	20.00	60.00	66.67	71.43	75.00	77.78
15	6.25	11.76	16.67	21.05	25.00	66.67	72.73	76.92	80.00	82.35
20	9.09	16.67	23.08	28.57	33.33	75.00	80.00	83.33	85.71	87.50
25	16.67	28.57	37.50	44.44	50.00	85.71	88.89	90.91	92.31	93.33

4EC (even room temperature has been acceptable). HSB is usually of acceptable quality even after several months in storage.

13.6.23.5 Heat Preparation of Brine

13.6.23.5.1 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.

13.6.23.5.2 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.

13.6.23.5.3 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‰ an that the temperature does not exceed 40EC. Additional seawater may be added to the brine to obtain the volume of brine required.

13.6.23.5.4 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.

13.6.23.6 Artificial Sea Salts

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

13.6.23.7 Dilution Water Preparation from Brine

13.6.23.7.1 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.

13.6.23.7.2 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 30‰, 100‰ divided by 30‰ = 3.33. The proportion of brine is 1 part in 3.33 (one part brine to 2.33 parts reagent water). To make 1 L of dilution water at 30‰ salinity from a HSB of 100‰, 300 mL of brine and 700 mL of reagent water are required. Verify the salinity of the resulting mixture using a refractometer.

13.6.23.8 Test Solution Salinity Adjustment

13.6.23.8.1 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.

13.6.23.8.2 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).

13.6.23.8.3 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:

$$VB = VE \times (30 - SE)/(SB - 30)$$

13.6.23.8.4 This calculation assumes that dilution water salinity is 30 ± 2‰.

13.6.23.9 Preparing Test Solution**s**

13.6.23.9.1 Ten mL of test solution are needed for each test container. To prepare test solutions at low effluent concentrations (<6%), effluents may be added directly to dilution water. For example, to prepare 1% effluent, add 1.0 mL of effluent to a 100-mL volumetric flask using a volumetric pipet or calibrated automatic pipet. Fill the volumetric flask to the 100-mL mark with dilution water, stopper it, and shake to mix. Pour into a (150-250 mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.9.2 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. Pour into a (100-250

TABLE 2. EXAMPLES OF EFFLUENT DILUTION SHOWING VOLUMES OF EFFLUENT (AT 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. Make serial dilutions from the highest test concentration.

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.

mL) beaker and stir. Distribute equal volumes into the replicate test chambers. The remaining test solution can be used for chemistry.

13.6.23.10 Brine Controls

13.6.23.10.1 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, 13.6.23.8.3) setting SE = 0, and solving for VE.

$$VE = VB \times (SB - 30)/(30 - SE)$$

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.

13.6.24 TEST ORGANISMS, OYSTERS AND MUSSELS

13.6.24.1 The test organisms for this test are the Pacific, oyster, *Crassostrea gigas,* or mussels, *Mytilus spp*. (at least twelve per test). Pacific oysters are native to Japan, but have been cultured commercially on the west coast of the United States for over a century.

13.6.24.2 Species Identification

13.6.24.2.1 The three species of mussels included in this method are presumably native to the west coast. The California mussel (*Mytilus californianus*) is distributed along the exposed rocky coast from Alaska to Baja California and is found from intertidal areas to 150 feet depth. The other two mussels included in this method (*M. trossulus* and *M. galloprovinciallis*) are common in sheltered waters such as bays and estuaries and were previously considered to be west coast populations of *Mytilus edulis*. The two species are both present in central California, with *M.*

galloprovincialis reported from San Francisco Bay to Baja California, and *M. trossulus* reported from Monterey to Alaska.

13.6.24.2.2 Test organisms should be identified to species using morphological features in recognized keys. Separation of the "*M. edulis*" complex, (*M. trossulus*, and *M. galloprovinciallis*) may not be possible without electrophoretic characterization. The geographic source of the *Mytilus spp*. broodstock must be reported.

13.6.24.3 Obtaining Broodstock

13.6.24.3.1 Adult oysters (*Crassostrea gigas*) and mussels (*Mytilus spp*.) can be obtained from commercial suppliers and the mussels can also be collected from the field. Organisms are best shipped in damp towels or seaweed and kept cool (4-12EC). Note: if practical, check the sex ratio of brood stock or request such information from a commercial supplier. A highly skewed sex ratio could result in poor embryo yield.

13.6.24.4 Broodstock Culture and Handling

13.6.24.4.1 The adult bivalves are maintained in glass aquaria or fiberglass troughs or tanks. These are supplied continuously (approximately 5 L/min) with natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded. Prior to spawning, the animals should be brushed or gently scraped to remove barnacles and other encrusting organisms; this alleviates problems of egg and sperm contamination, especially through potential barnacle spawning.

13.6.24.4.2 Although ambient temperature seawater is usually acceptable for holding, recommended temperatures are 14-15EC for oyster and 8EC for mussels; conditioning bivalves to spawning condition usually requires holding for from 1-8 weeks at a higher temperature (20EC for oysters, 15-18EC for mussels).

13.6.24.4.3 Natural seawater ($30‰) is used to maintain the adult animals and as a control water in the tests.

13.6.24.4.4 Adult animals used in field studies are transported in insulated boxes or coolers packed with wet kelp or paper

toweling. Upon arrival at the field site, aquaria are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to the holding temperature before animals are added.

13.7 **EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE**

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

13.8 **CALIBRATION AND STANDARDIZATION**

13.8.1 See Section 4, Quality Assurance.

13.9 **QUALITY CONTROL**

13.9.1 See Section 4, Quality Assurance.

13.10 **TEST PROCEDURES**

13.10.1 TEST DESIGN

13.10.1.1 The test consists of at least four replicates of five effluent concentrations plus a dilution water control. Tests that use brine to adjust salinity must also contain four replicates of a brine control. In addition, at least six extra count controls are prepared in dilution water and the number of embryos in each are counted at the time of test initiation. These counts provide an average initial embryo density that is used in the calculation of test results (see 13.13.1.3). Extra replicates are recommended for water chemistry during the tests (see Section 13.8 and Table 3).

13.10.1.2 Effluent concentrations are expressed as percent effluent.

13.10.2 TEST SOLUTIONS

13.10.2.1 Receiving waters

13.10.2.1.1 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 four replicate chambers per test, each containing 10 mL, and 400 mL for chemical analysis, would require approximately 440 mL of sample per test.

13.10.2.2 Effluents

14.10.2.2.1 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 70% at 30‰ salinity.

13.10.2.2.2 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%).

13.10.2.2.3 The volume in each test chamber is 10 mL.

13.10.2.2.4 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.

13.10.2.3 Dilution Water

13.10.2.3.1 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.

13.10.2.4 Reference Toxicant Test

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

13.10.2.4.2 The preferred reference toxicant for oysters and mussels is copper chloride ($CuCl_2 \circ H_2O$). 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 copper reference toxicant stock solution (2,000 mg/L) by adding 5.366 g of copper chloride ($CuCl_2 \circ 2H_2O$) to 1 liter of reagent water. For each reference toxicant test prepare a copper sub-stock of 3 mg/L by diluting 1.5 mL of stock to one liter with reagent water. Alternatively, certified standard solutions can be ordered from commercial companies.

13.10.2.4.3 Prepare a control (0 Fg/L) plus four replicates each of at least five consecutive copper reference toxicant solutions (e.g., from the series 3.0, 4.4, 6.5, 9.5, 13.9, 20.4, and 30.0 Fg/L, by adding 0.10, 0.15, 0.22, 0.32, 0.46, 0.68, and 1.00 mL of sub-stock solution, respectively, to 100-mL volumetric flasks and filling to 100-mL with dilution water). Start with control solutions and progress to the highest concentration to minimize contamination.

13.10.2.4.4 If the effluent and reference toxicant tests are to be run concurrently, then the tests must use embryos from the same spawn. 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 30 ± 2‰.

13.10.3 COLLECTION OF GAMETES FOR THE TEST

13.10.3.1 Spawning Induction

13.10.3.1.1 Select at least a dozen bivalves and place them into a container filled with seawater (ca. 20EC for oysters, 15EC for

mussels) and allow time for them to resume pumping (ca. 30 minutes). Mussels will often start pumping following immersion if they have been kept out of water and refrigerated overnight prior to spawning.

TABLE 3. EXAMPLE OF TYPICAL TEST ARRAY SHOWING NUMBER AND TYPES OF TREATMENT CHAMBERS REQUIRED.

TREATMENT	Test Vials	Chemistry Vials
Count Control	6	0
Brine Control	4	1-3
Dilution Water Control	4	1-3
Effluent conc. 1	4	1-3
Effluent conc. 2	4	1-3
Effluent conc. 3	4	1-3
Effluent conc. 4	4	1-3
Effluent conc. 5	4	1-3
TOTAL Chambers = 41-55	34	7-21

13.10.3.1.2 Over a 15-20 minute period, increase the temperature (do not exceed 32EC for oysters, or 20EC for mussels), checking for spawning.

13.10.3.1.3 If no spawning occurs after 30 minutes, replace the water with some at the original temperature and after 15 minutes again increase the temperature as in 13.10.3.2. Although ASTM (1993) cautions against it, the addition of algae into the water can often stimulate spawning of bivalves; if this method is used, the organisms should be moved to clean water once spawning begins. Mussels can also be induced to spawn by injection of 0.5 M KCl into the posterior adductor muscle. Oysters can be induced to spawn by the addition of heat-killed sperm about one hour after initial temperature increase.

13.10.3.2 Pooling Gametes

13.10.3.2.1 When individuals are observed to be shedding gametes, remove each spawner from the tank and place each in a separate container (20EC water for oysters, 15EC for mussels). Alternatively, bivalves can be placed into individual chambers initially (at temperatures per 13.10.5.2) and these placed into a water bath that provides the desired maximum temperature.

13.10.3.2.2 Early in the spawning process, examine a small sample of the gametes from each spawner to confirm sex and to see if the gametes are of adequate quality.

13.10.3.2.3 Place a small amount of sperm from each male onto a microscope slide (well slides work nicely). Examine the sperm for motility; use sperm from those males with the better sperm motility.

13.10.3.2.4 A small sample of the eggs from each female should be examined for the presence of significant quantities of poor eggs (vacuolated, small, or abnormally shaped). If good quality eggs are available from one or more females, questionable batches of eggs should not be used for the test. It is more important to use high quality eggs than it is to use a pooled population of eggs.

13.10.3.2.5 Sperm and egg suspensions that are to be used for preparing the embryo stock should be passed through Nytex screen (ca. 75 Fm) to separate out clumps of gametes or extraneous material.

13.10.3.2.6 The pooled eggs are placed into a 1 L beaker and sufficient dilution water added to achieve an egg density of about 5,000-8,000 eggs/mL (objects are just discernible when viewed through the egg suspension) in about 800-900 mL water volume.

13.10.3.3 Fertilization

13.10.3.3.1 Sperm density may vary from one spawning to the next. It is important to use enough sperm to achieve a high percent egg fertilization, but too many sperm can cause polyspermy with resultant abnormal development. To achieve an acceptable level of sperm, several egg suspensions of equal density should be fertilized using a range of sperm volumes,

e.g., 100 mL of egg suspension plus 1, 3, and 10 mL of sperm suspension. This test fertilization should be accomplished within 1 hour of spawning. Use the eggs with the lowest amount of sperm giving normal embryo development after 1.5-2.5 hours after fertilization, as determined by microscopic examination. Usually >90% of the eggs should be fertilized; oysters should have changed from the tear-drop shaped egg to a round single cell zygote; mussels should show a single polar body; or embryos of either species should have advanced to the two-cell stage.

13.10.4 START OF THE TEST

13.10.4.1 Prior to Beginning the Test

13.10.4.1.1 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).

13.10.4.1.2 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 (18 or 20 ± 1EC) and maintained at that temperature during the addition of dilution water.

13.10.4.1.3 Increase the temperature of the water bath, room, or incubator to the required test temperature (18 or 20 ± 1EC).

13.10.4.1.4 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 oysters or mussels have been examined at the end of the test.

13.10.4.1.5 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.

13.10.4.1.6 Arrange the test chambers randomly in the water bath or controlled temperature room. Once chambers have been labeled randomly, they can be arranged in numerical order for convenience, since this will also ensure random placement of treatments.

13.10.4.2 Estimation of Embryo Density

13.10.4.2.1 Adjust the embryo suspension to a density of 1,500-3,000/mL. Confirm by counting chamber counts on 1 mL subsamples from a stirred suspension of embryos. Final larval density of 15/mL will provide reasonable precision (150 larvae) and be easier to count than 300 larvae. Add 0.1 mL of the embryo suspension to 10 mL of test solution into each of the randomized test vials. It is extremely important (for a consistent embryo density among test chambers) to maintain a homogeneous distribution of embryos in the stock suspension by regular, slow oscillation of a perforated plunger during embryo distribution.

13.10.4.3 Initial Density Counts

13.10.4.3.1 If tests are conducted on small volumes, using an inverted microscope, the total number of embryos injected into the count controls should be determined as soon as the test has been started. If larger test volumes are used, with counts based upon subsamples, the embros should be resuspended in the water using a perforated plunger. Then subsamples are taken (e.g., 5-10 mL) and the total number of embryos counted in the subsample. Two methods for these counts are to use a counting chamber of the same volume as the subsample, or to screen the embryos using a 37 Fm screen and backwash with a smaller volume for small counting chambers. In either procedure, appropriate multiple rinsing is needed to achieve quantitative transfer of embryos.

13.10.4.3.2 Initial counts are required to determine survival in the controls and other treatments. High coefficients of variability in initial counts make survival estimates inexact and may actually decrease the sensitivity of the test.

13.10.4.4 Incubation

13.10.4.4.1 Cover and incubate the chambers in an environmental chamber or by partial immersion in a temperature-controlled water bath for 48 hours.

13.10.4.4.2 At the end of the 48-hour incubation period, examine a count control test chamber (or control test vial if the count controls were transferred to a counting chamber to make the initial counts) under a microscope to check for complete development of control organisms. If development is complete, the test should be ended. If development does not appear to be complete, the test should be continued until complete development occurs (but not beyond 54 hours total test duration).

13.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

13.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 µE/m2/s, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

13.10.5.2 The water temperature in the test chambers should be maintained at 18 or 20 ± 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.

13.10.5.3 The test salinity should be in the range of 30 ± 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.

13.10.5.4 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.

13.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

13.10.6.1 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.

13.10.7 OBSERVATIONS DURING THE TEST

13.10.7.1 Routine Chemical and Physical Observations

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

13.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in each test 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.

13.10.7.1.3 Record all the measurements on the data sheet.

13.10.8 TERMINATION OF THE TEST

13.10.8.1 Ending the Test

13.10.8.1.1 Record the time the test is terminated.

13.10.8.1.2 The 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. If small electrodes are used, these measurements can be performed in a single extra replicate vial set up specifically for this measurement. Measurements should not be made in vials that are to be counted, as larvae may adhere to electrodes, possibly biasing larval counts.

13.10.8.2 Sample Preservation

13.10.8.2.1 To terminate the test, add 0.25 mL of concentrated formalin (37% formaldehyde). It is advisable not to shake the contents at any time following test termination because the larvae may stick to the edge of the chambers. Simply allow the preservative to mix passively and the larvae to settle out. The use of glutaraldehyde instead of formalin is likely to be acceptable, but as no record of its use with this test is known, care should be taken to confirm that glutaraldehyde kills, preserves, and produces no artifacts that would confound the test results.

13.10.8.2.2 Note: Formaldehyde has been identified as a carcinogen and both glutaraldehyde and formaldehyde are irritating to skin and mucus membranes. Neither should be used at higher concentrations than needed to achieve morphological preservation and only under conditions of maximal ventilation and minimal opportunity for volatilization into room air.

13.10.8.3 Counting

13.10.8.3.1 After addition of preservative, observe all the larvae in each test vial. This can be done by examining the contents of each test vial with an inverted microscope at about 40X-50X magnification or by quantitative transfer of all larvae onto a counting chamber and counting using a compound microscope at about 100X. Using the mechanical stage, carefully count and score all larvae as either normal or abnormal. If substantial numbers of completely developed shells without meat are observed (i.e., > 5 percent of normal larvae), then these shells should be enumerated separately (as dead larvae). "Larvae possessing misshapened or otherwise malformed shells are considered normal, provided development has been completed" (ASTM, 1994). Record the final counts on the data sheet.

13.10.8.3.2 If the number of larvae observed appears to be low in relation to the number inoculated at the beginning of the test, this signifies either mortality and dissolution, or possible adherence to the walls of the vials or incomplete transfer to the counting chamber. Inspect the vials for evidence of the latter two occurrences.

13.10.8.4 Endpoint

13.10.8.4.1 The percentage of embryos that did not survive and develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (See 13.13.1.3). All larvae are considered live unless they are merely empty shells "without meat" (ASTM, 1994); embryos and larvae that are not yet in the D-hinge stage are counted as abnormal, even if they may have died during the test. Embryos and larvae that die and disintegrate during the test are estimated from initial embryo counts (See N' in 13.13.1.3).

13.10.8.4.2 Unless used as the dilution water, natural seawater controls are only used to check the relative performance of the dilution water controls (e.g., brine controls) required for salinity adjustment. Statistical analysis should use the appropriate dilution water control data.

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

13.11.1 A summary of test conditions and test acceptability criteria is listed in Table 4.

TABLE 4. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *CRASSOSTREA GIGAS* and *MYTILUS SPP*., EMBRYO-LARVAL DEVELOPMENT TEST WITH EFFLUENTS AND RECEIVING WATERS

1. Test type:	Static non-renewal
2. Salinity:	30 ± 2‰
3. Temperature:	20 ± 1EC (oysters) 15 or 18 ± 1EC (mussels)*
4. Light quality:	Ambient laboratory light

5. Light intensity:	l0-20 uE/m2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	30 mL
8. Test solution volume:	10 mL
9. No. larvae per chamber:	150-300
10. No. replicate chambers per concentration:	4 (plus 3 chemistry vials)
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 hours (or until complete development up to 54 hours)
15. Endpoint:	Survival and normal shell development
16. Test acceptability criteria:	Control survival must be $70% for oyster embryos or $50% for mussel embryos in control vials; $90% normal shell development in surviving controls; and must achieve a %MSD of <25%

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:	1 L per test

*Mussel embryo-larval tests were commonly conducted at 15EC (ASTM, 1994). Experience has shown that many laboratories in northern Californa, Oregon, and Washington often fail to achieve adequate control development at 15EC in 48 hours. It is acceptable to conduct the test at 15EC with the permission of the regulatory authority. Developmental rates may be dependent upon species, local population characteristics, or other factors.

13.12 **ACCEPTABILITY OF TEST RESULTS**

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

(1) The mean survival must be at least 70% for oysters or at least 50% for mussels in the controls.

(2) The percent normal must be at least 90% in the surviving controls.

(3) The minimum significant difference (%MSD) is <25% relative to the control.

13.13 **DATA ANALYSIS**

13.13.1 **GENERAL**

13.13.1.1 Tabulate and summarize the data. Calculate the proportion of normally developed larvae for each replicate. A sample set of test data is listed in Table 5.

13.13.1.2 Final calculations are based upon counts of normal larvae and total larvae at test termination, and mean initial embryo count.

13.13.1.3 The percentage of embryos that did not survive or develop to live larvae with completely developed shells (i.e., abnormal or dead organisms) is calculated for each treatment replicate (including controls) using the formula:

$$A = \frac{100 (N' - B')}{N'}$$

where:

A = percent abnormal and dead organisms
B' = the adjusted number of normal larvae at the end of the test
N' = the initial number of embryos in the test chambers expressed as the mean of the initial counts; and: if N > N', where

N = the actual number of larvae at the end of the test then: $B' = B (N' / N)$

where: B = the actual number of normal larvae at the end of the test but, when N # N', then: $B' = B$

The means of "A" are obtained for each treatment concentration, and the latter are corrected for control response using Abbott's formula, as follows:

$$E = \frac{100 (A - M)}{100 - M}$$

where:

E = the mean percent abnormal/dead corrected for controls
A = the mean percent abnormal/dead
M = the value of A for the controls.

13.13.1.4 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.

TABLE 5. DATA FROM BIVALVE DEVELOPMENT TEST

Copper Concentration (µg/L)	Replicate	Initial Density	Number Surviving	Number Normal	Proportion Normal
Control	A	25	22	22	1.00
	B	25	25	24	0.96
	C	25	25	25	1.00
	D	30	30	29	0.97
0.13	A	25	23	22	0.96
	B	30	30	29	0.97
	C	25	25	25	1.00
	D	25	24	23	0.96
0.25	A	25	25	23	0.92
	B	25	19	18	0.95
	C	25	21	19	0.90
	D	25	23	22	0.96
0.50	A	25	11	10	0.91
	B	25	14	13	0.93
	C	25	17	15	0.88
	D	25	15	14	0.93
1.00	A	25	8	7	0.88
	B	25	6	5	0.83
	C	25	8	7	0.88
	D	25	11	9	0.82
2.00	A	25	2	2	1.00
	B	25	3	2	0.67
	C	25	4	3	0.75
	D	25	5	2	0.40

13.13.1.5 The endpoints of toxicity tests using bivalves are based on the reduction in proportion of normally developed larvae. The IC25 is calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for larval development 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. See the Appendices for examples of the manual computations, and examples of data input and program output.

13.13.2 EXAMPLE OF ANALYSIS OF BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

13.13.2.1 Formal statistical analysis of the embryo-larval development is outlined in Figure 1. The response used in the analysis is the proportion of normally developed surviving larvae 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 normal development in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25.

13.13.2.2 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.

13.13.2.3 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.

13.13.2.4 Example of Analysis of Embryo-Larval Development Data

13.13.2.4.1 Since the response of interest is the proportion of normally developed surviving larvae, each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because there are varying numbers of survivors in the replicates, the adjustment for response proportions of zero or one will not be made. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 5. The data are plotted in Figure 2.

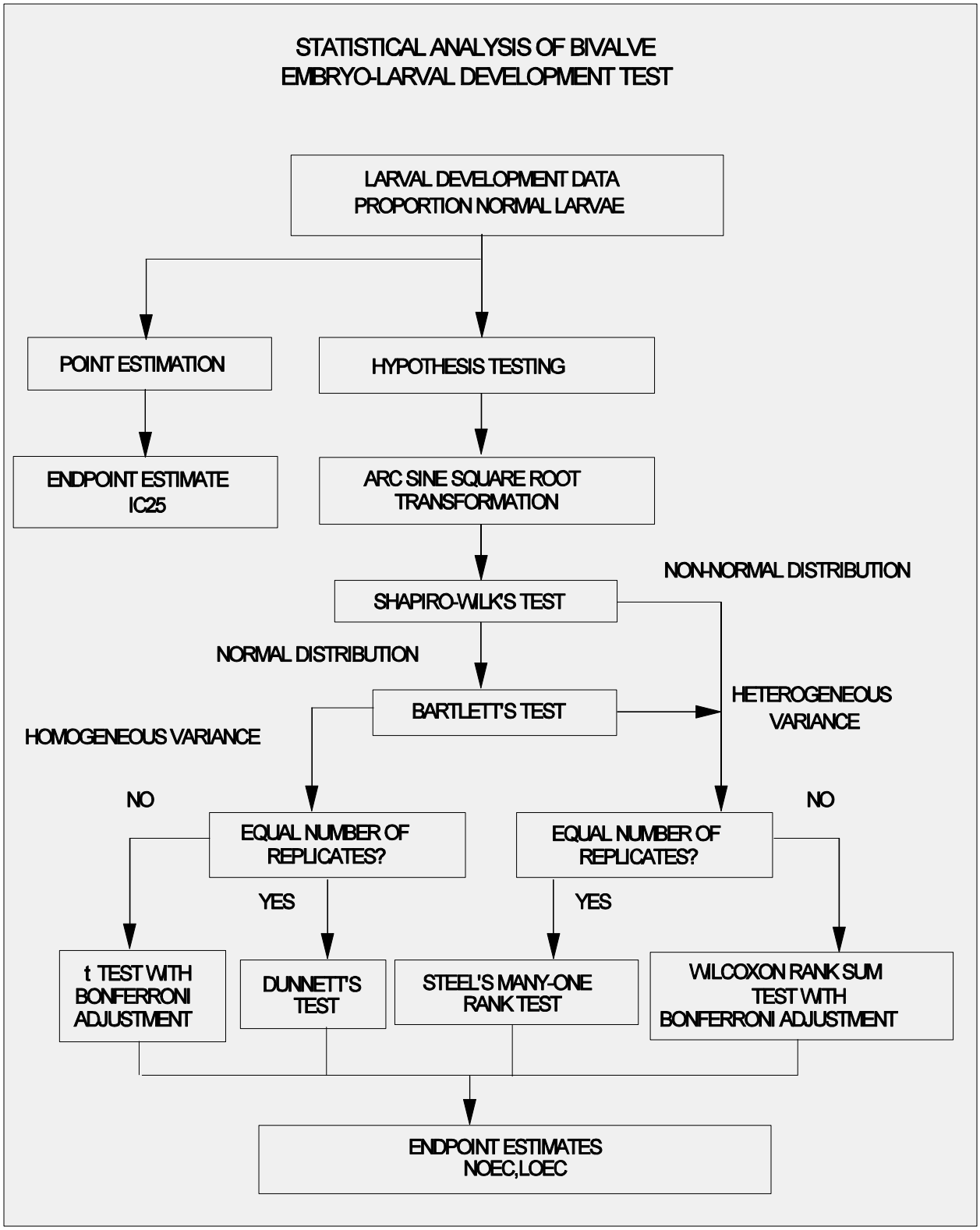

Figure 1. Flowchart for statistical analysis of the pacific oyster, *Crassostrea gigas*, and mussel, *Mytilus spp.*, development data.

13.13.2.5 Test for Normality

13.13.2.5.1 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 summarized in Table 6.

TABLE 6. BIVALVE EMBRYO-LARVAL DEVELOPMENT DATA

		Copper Concentration (µg/L)					
	Replicate	Control	0.13	0.25	0.50	1.00	2.00
RAW	A	1.00	0.96	0.92	0.91	0.88	1.00
	B	0.96	0.97	0.95	0.93	0.83	0.67
	C	1.00	1.00	0.90	0.88	0.88	0.75
	D	0.97	0.96	0.96	0.93	0.82	0.40
ARC SINE SQUARE ROOT TRANSFORMED	A	1.571	1.369	1.284	1.266	1.217	1.571
	B	1.369	1.397	1.345	1.303	1.146	0.959
	C	1.571	1.571	1.249	1.217	1.217	1.047
	D	1.397	1.369	1.369	1.303	1.133	0.685
Mean ($\bar{X}_i$)		1.477	1.427	1.312	1.272	1.178	1.066
S_i^2		0.01191	0.00945	0.00303	0.00166	0.00203	0.13733
i		1	2	3	4	5	6

13.13.2.5.2 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

13.13.2.5.3 For this set of data, n = 24

$$\bar{X} = \frac{1}{24}(-0.002) = 0.000$$

D = 0.4963

13.13.2.5.4 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 7

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

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.381	13	-0.019
2	-0.108	14	-0.006
3	-0.107	15	0.031
4	-0.080	16	0.031
5	-0.063	17	0.033
6	-0.058	18	0.039
7	-0.058	19	0.039
8	-0.055	20	0.057
9	-0.045	21	0.094
10	-0.032	22	0.094
11	-0.030	23	0.144
12	-0.028	24	0.505

13.13.2.5.5 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 = 24 and k = 12. The a_i values are listed in Table 8.

13.13.2.5.6 Compute the test statistic, W, as follows:

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

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

$$W = \frac{1}{0.4963} (0.6322)^2 = 0.805$$

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

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.886	$X^{(24)} - X^{(1)}$
2	0.3098	0.252	$X^{(23)} - X^{(2)}$
3	0.2554	0.201	$X^{(22)} - X^{(3)}$
4	0.2154	0.174	$X^{(21)} - X^{(4)}$
5	0.1807	0.120	$X^{(20)} - X^{(5)}$
6	0.1512	0.097	$X^{(19)} - X^{(6)}$
7	0.1245	0.097	$X^{(18)} - X^{(7)}$
8	0.0997	0.088	$X^{(17)} - X^{(8)}$
9	0.0764	0.076	$X^{(16)} - X^{(9)}$
10	0.0539	0.063	$X^{(15)} - X^{(10)}$
11	0.0321	0.024	$X^{(14)} - X^{(11)}$
12	0.0107	0.009	$X^{(13)} - X^{(12)}$

13.13.2.5.7 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 the data in this example, the critical value at a significance level of 0.01 and n = 24 observations is 0.884. Since W = 0.805 is less than the critical value, conclude that the data are not normally distributed.

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

13.13.2.6 Steel's Many-one Rank Test

13.13.2.6.1 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, ... , 8) 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.

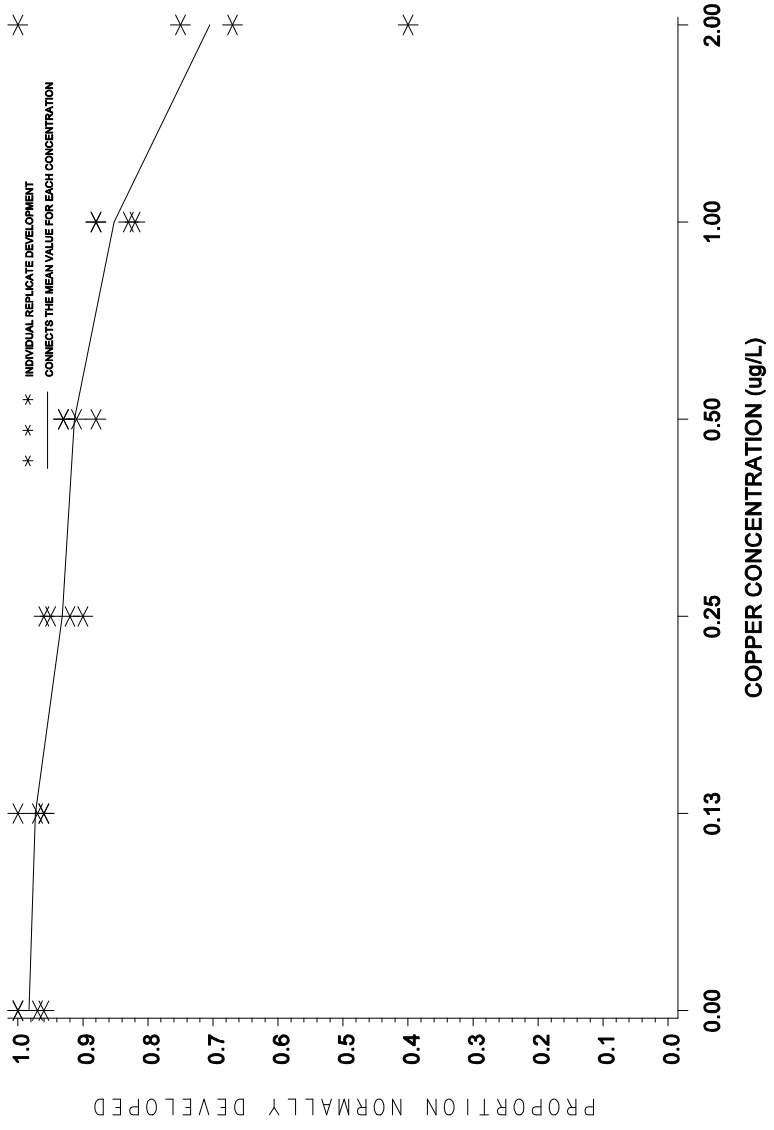

Figure 2. Plot of mean proportion of normaly developed bivalve larvae.

13.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.13 µg/L 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.

TABLE 9. ASSIGNING RANKS TO THE CONTROL AND 0.13 µg/L CONCENTRATION LEVEL FOR STEEL'S MANY-ONE RANK TEST

Rank	Transformed Proportion Normal	Concentration
2	1.369	0.13 µg/L
2	1.369	0.13 µg/L
2	1.369	Control
4.5	1.397	0.13 µg/L
4.5	1.397	Control
7	1.571	0.13 µg/L
7	1.571	Control
7	1.571	Control

13.13.2.6.3 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 five concentrations (excluding the control) and four replicates is 10 (See Table 5, Appendix E).

13.13.2.6.4 Since the rank sums for the 0.50 µg/L and 1.00 µg/L concentration levels are equal to the critical value, the proportions of normal development in those concentrations are considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no

TABLE 10. TABLE OF RANKS[1]

Replicate	Control	Copper Concentration (µg/L) 0.13	0.25
1	1.571(7,7.5,7.5,7.5,7)	1.369(2)	1.284(2)
2	1.369(2,4.5,5,5,4)	1.397(4.5)	1.345(3)
3	1.571(7,7.5,7.5,7.5,7)	1.571(7)	1.249(1)
4	1.397(4.5,6,6,6,5)	1.369(2)	1.369(4.5)

Replicate	Copper Concentration (µg/L) (Continued) 0.50	1.00	2.00
1	1.266(2)	1.217(3.5)	1.571(7)
2	1.303(3.5)	1.146(2)	0.959(2)
3	1.217(1)	1.217(3.5)	1.047(3)
4	1.303(3.5)	1.133(1)	0.685(1)

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

TABLE 11. RANK SUMS

Concentration µg/L Copper)	Rank Sum
0.13	15.5
0.25	10.5
0.50	10.0
1.00	10.0

other concentration has a significantly lower proportion normal than the control. Because the 0.50 µg/L concentration shows significantly lower normal development than the control while the

higher 2.00 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 0.25 µg/L and 0.50 µg/L, respectively.

13.13.2.7 Calculation of the ICp

13.13.2.7.1 The embryo-larval development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 2, the observed means are monotonically non-increasing with respect to concentration (mean response for each higher concentration is not less than or equal to the mean response for the previous concentration and the responses between concentrations do not follow a linear trends). Therefore, it is not necessary to smooth the means prior to calculating the IC. The observed means, represented by $\&_i$ become the corresponding smoothed means, M_i. Table 12 contains the response means and smoothed means and Figure 3 gives a plot of the smoothed response curve.

TABLE 12. BIVALVE MEAN LARVAL DEVELOPMENT RESPONSE AFTER SMOOTHING

Copper Conc. (µg/L)	i	Response Means, $\&_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.983	0.983
0.13	2	0.973	0.973
0.25	3	0.932	0.932
0.50	4	0.913	0.913
1.00	5	0.852	0.852
2.00	6	0.705	0.705

13.13.2.7.2 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of normally developed larvae, compared to the controls, would result

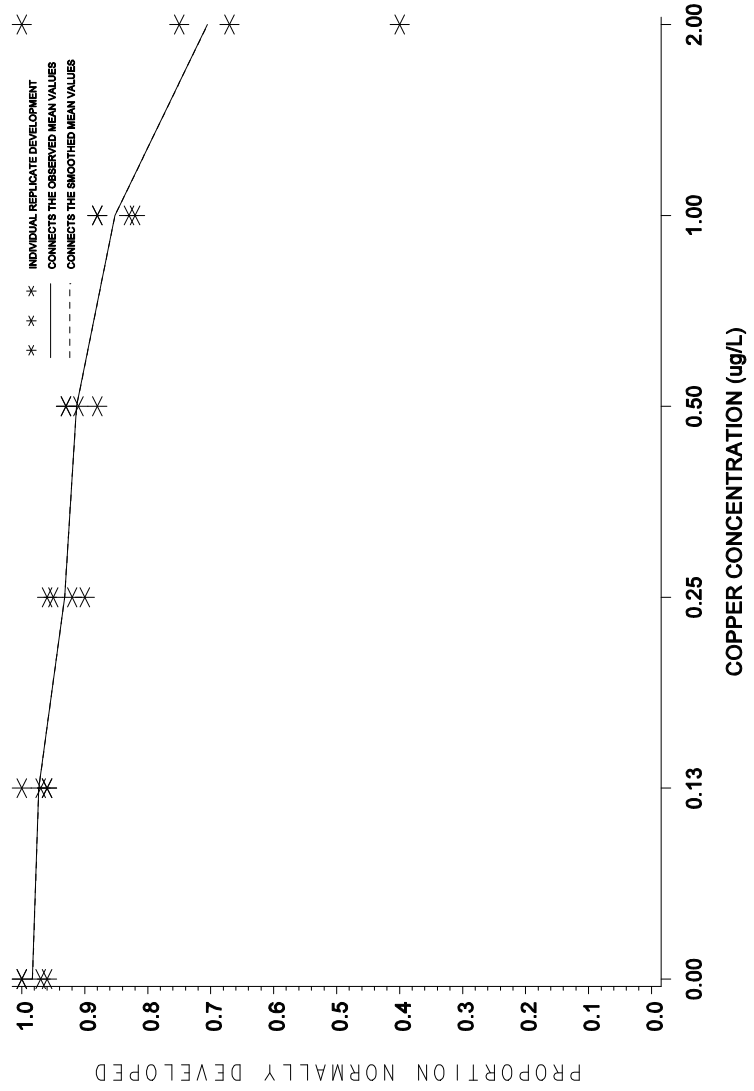

Figure 3. Plot of raw data, observed means, and smoothed means for the bivalve development data from Tables 4 and 12.

in a mean proportion of 0.737, where $M_1(1-p/100) = 0.983(1-25/100)$. Examining the means and their associated concentrations (Table 12), the response, 0.737, is bracketed by C_4 = 1.00 µg/L copper and C_5 = 2.00 µg/L copper.

13.13.2.7.3 Using the equation from Section 4.2 in 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 = 1.00 + [0.983(1 - 25/100) - 0.852] (2.00 - 1.00)
S))))))))))))Q
(0.705 - 0.852)
= 1.78 µg/L.

13.13.2.7.4 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 1.7839 µg/L. The empirical 95.0% confidence interval for the true mean was not available because the number of resamples which generated an IC25 estimate was not an even multiple of 40. The computer program output for the IC25 for this data set is shown in Figure 4.

13.14 **PRECISION AND ACCURACY**

13.14.1 PRECISION

13.14.1.1 Single-Laboratory Precision

13.14.1.1.1 Single-laboratory precision data for the *Mytilus spp*. with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 4-5. The coefficient of variation, based on EC25, is 32.8% to 45.0% for cadmium and 14.2% to 30.6% for lyophilized pulp mill effluent. Single-laboratory precision data for the *Crassostrea gigas* with the reference toxicant cadmium and lyophilized pulp mill effluent with natural seawater are provided in Tables 6-7. The coefficient of variation, based on EC25, is 18.5% to 80.4% for cadmium and 20.8% to 43.3% for lyophilized pulp mill effluent.

13.14.1.2 Multi-laboratory Precision

```
Conc. ID             1         2         3         4         5         6
-------------------------------------------------------------------------
Conc. Tested         0      0.13      0.25      0.50      1.00      2.00
-------------------------------------------------------------------------
Response  1       1.00      0.96      0.92      0.91      0.88      1.00
Response  2       0.96      0.97      0.95      0.93      0.83      0.67
Response  3       1.00      1.00      0.90      0.88      0.88      0.75
Response  4       0.97      0.96      0.96      0.93      0.82      0.40
-------------------------------------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date:    Test Ending Date:
Test Species: bivalve
Test Duration:        48 hours
DATA FILE: bivalve.icp
OUTPUT FILE: bivalve.i25
-------------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates            ug/L       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          4             0.000          0.983      0.021      0.983
  2          4             0.130          0.973      0.019      0.973
  3          4             0.250          0.932      0.028      0.932
  4          4             0.500          0.913      0.024      0.913
  5          4             1.000          0.852      0.032      0.852
  6          4             2.000          0.705      0.247      0.705
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     1.7839   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80Those resamples not used had estimates
above the highest concentration/ %Effluent.

The Bootstrap Estimates Mean:   1.6188 Standard Deviation:     0.1758

No Confidence Limits can be produced since the number of resamples
generated is not a multiple of 40.
Resampling time in Seconds:     0.17  Random_Seed: -232404862
```

Figure 4. ICPIN program output for the IC25.

13.14.1.2.1 Multi-laboratory precision data for *Mytilus spp*. with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 12-13. The coefficient of variation for cadmium EC25 is 23.6%, and for effluent EC25 is 14.4% based on five laboratories. Multi-laboratory precision data for *Crassostrea gigas* with the reference toxicant, cadmium and lyophilized pulp mill effluent are provided in Tables 14-15. The coefficient of variation is 21.3% for cadmium EC25 and 14.2% for lyophilized pulp mill effluent EC25, based on results from five laboratories.

13.14.2 ACCURACY

13.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 12. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, *MYTILUS SPP*., DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	2.35	1.06	2.42	4.20	4.77
Nov-92	0.86	3.49	3.89	2.21	2.39
Dec-92	1.79	2.51	no data	2.27	3.73
Jan-93	3.69	2.25	6.77	no data	1.57
Feb-93	2.81	2.91	5.85	3.75	3.05
Mar-93	3.71	2.64	2.62	4.89	no data

Mean	2.54	2.48	4.31	3.46	3.10
SD	1.11	0.81	1.94	1.19	1.23
CV (%)	43.9	32.8	45.0	34.3	40.0

# of Labs	Statistic	EC25
5	Mean (N=5) SD CV(%)	3.18 0.75 23.6

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 13. SINGLE AND MULTI-LABORATORY PRECISION OF THE MUSSEL, *MYTILUS SPP.*, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
Oct-92	1.78	1.40	2.02	1.83	1.85
Nov-92	1.57	1.94	2.70	1.98	no data
Dec-92	1.74	1.88	3.08	no data	1.87
Jan-93	3.17	2.03	2.46	1.07	no data
Feb-93	1.66	no data	no data	no data	no data
Mar-93	1.85	1.66	1.72	1.82	no data

Mean	1.96	1.78	2.40	1.68	1.86
SD	0.60	0.25	0.54	0.41	0.28
CV (%)	30.6	14.2	22.5	24.5	1.4

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.93 0.28 14.4

These data are from: Pastorok, et al. (1994) West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 14. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, *CRASSOSTREA GIGAS*, DEVELOPMENT TEST PERFORMED WITH CADMIUM CHLORIDE (CD MG/L) AS A REFERENCE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	1.04	1.54	0.50	0.41	0.56
Aug-92	0.31	1.38	0.30	0.35	no data
Sept-92	0.68	0.20	0.49	no data	no data
Apr-93	no data	0.45	0.51	no data	0.95
May-93	0.46	0.30	1.05	0.52	0.83
June-93	0.26	1.55	0.93	no data	0.83
July-93	0.28	0.82	0.66	1.56	0.90

Mean	0.51	0.89	0.63	0.71	0.81
SD	0.31	0.59	0.27	0.57	0.15
CV (%)	60.6	66.7	42.1	80.4	18.5

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	0.71 0.15 21.3

These data are from: Pastorok, et al. (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

TABLE 15. SINGLE AND MULTI-LABORATORY PRECISION OF THE OYSTER, *CRASSOSTREA GIGAS*, DEVELOPMENT TEST PERFORMED WITH LYOPHILIZED PULP MILL EFFLUENT (%) AS THE TOXICANT

Month	Lab A	Lab B	Lab C	Lab D	Lab E
July-92	no data	0.91	1.28	no data	1.43
Aug-92	1.21	1.09	0.98	0.61	no data
Sept-92	0.76	1.66	0.83	no data	no data
Apr-93	0.80	1.10	1.61	1.66	no data
May-93	1.21	0.65	1.90	0.93	0.93
June-93	1.09	1.32	1.72	0.83	0.98
July-93	0.82	0.80	1.56	1.67	1.04

Mean	0.98	1.08	1.41	1.14	1.10
SD.	0.21	0.34	0.40	0.49	0.23
CV (%)	21.6	31.4	28.0	43.3	20.8

# of Labs	Statistic	EC25
5	Mean (n=5) SD CV(%)	1.14 0.16 14.2

These data are from: Pastorok, et al., (1994), West Coast Marine Species Chronic Protocol Variability Study, PTI Environmental Services, Prepared for Washington Department of Ecology, February, 1994.

APPENDIX I. BIVALVE 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 30 ± 2‰. Include brine controls in tests that use brine.

C. Prepare a copper reference toxicant stock solution.

D. Prepare a series copper reference toxicant concentrations.

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 18 or 20EC 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. Determine test concentrations and appropriate dilution water based on NPDES permit requirements and guidance from the appropriate regulatory agency.

B. Prepare test solutions by diluting well mixed unfiltered effluent using volumetric pipettes. Use hypersaline brine where necessary to maintain all test solutions at 30 ± 2‰. Include brine controls in tests that use brine.

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

D. 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.

E. Place test chambers in a water bath or environmental chamber set to 18 or 20EC as appropriate for the test species and allow temperature to equilibrate.

F. Measure the test solution temperature daily in a randomly located blank test chamber. Monitor the temperature of the water bath or environmental chamber continuously.

PREPARATION AND ANALYSIS OF TEST ORGANISMS

A. Obtain test organisms and hold or condition as necessary for spawning.

B. On day of test, spawn organisms, examine gametes, pool good eggs, pool good sperm.

C. Fertilize subsets of eggs with a range of sperm concentrations to obtain >90% embryogenesis without polyspermy.

D. Adjust embryo stock suspension density to 1500-3000/mL.

E. Introduce organisms to test chambers (150-300 embryos in 0.1 mL of stock).

F. Count all embryos in each of six extra controls set up for determining mean embryo density and variation. Return these to the test for later examination for developmental rate in controls.

G. Near the end of the 48-hour incubation period examine several of the extra controls to determine if development has reached the prodisoconch stage. If yes, terminate the test at 48 hours; if no, continue the test for up to 54 hours as required for complete development.

H. Terminate the test by addition of formalin.

I. Count larvae and record the number of normal prodisoconch larvae and other larvae in each test vial.

J. Analyze the data.

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

Sample data sheet for embryo microscopic examination.

BIVALVE DEVELOPMENT TEST: RESULTS

Bioassay No._____ Date_____

Counter_____

Number	Sample	Abnormal	Normal	%Normal	Notes