SECTION 14

RED ABALONE, *Haliotis rufescens* LARVAL DEVELOPMENT TEST METHOD

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RED ABALONE, *HALIOTUS RUFESCENS*
LARVAL DEVELOPMENT TEST METHOD

14.1 SCOPE AND APPLICATION

14.1.1 This method estimates the chronic toxicity of effluents and receiving waters to the larvae of red abalone, *Haliotis rufescens* during a 48-h static non-renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

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

14.1.3 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.

14.1.4 This method 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.

14.1.5 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.

14.2 SUMMARY OF METHOD

14.2.1 This method provides the step-by-step instructions for performing a 48-h static non-renewal test using early development of abalone larvae to determine the toxicity of substances in marine and estuarine waters. The test endpoint is normal shell development.

14.3 **INTERFERENCES**

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

14.3.2 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).

14.4 **SAFETY**

14.4.1 See Section 3, Health and Safety.

14.5 **APPARATUS AND EQUIPMENT**

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

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

14.5.3 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.

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

14.5.5 Refractometer -- for determining salinity.

14.5.6 Hydrometer(s) -- for calibrating refractometer.

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

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

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

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

14.5.11 Winkler bottles -- for dissolved oxygen determinations.

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

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

14.5.14 Glass stirring rods -- for mixing test solutions.

14.5.15 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).

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

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

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

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

14.5.20 Wash bottles -- for dilution water.

14.5.21 20-liter cubitainers or polycarbonate water cooler jugs -- for making hypersaline brine.

14.5.22 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.

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

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

14.5.25 Counter, two unit, 0-999 -- for recording counts of larvae.

14.5.26 Inverted or compound microscope -- for inspecting gametes and making counts of larvae.

14.5.27 Perforated plunger -- for stirring egg solutions.

14.5.28 Supply of *Macrocystis* or other macroalgae (if holding broodstock for longer than 5 days) -- for feeding abalone.

14.5.29 Stainless steel butter knife, rounded smooth-edged blade (for handling adult abalone). Abalone irons and plastic putty knives have also been used successfully.

14.5.30 Sieve or screened tube, approximately 37 µm-mesh -- for retaining larvae at the end of the test.

14.5.31 60 µm NITEX® filter -- for filtering receiving water.

14.6 **REAGENTS AND SUPPLIES**

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

14.6.2 Data sheets (one set per test) -- for data recording (See Appendix I).

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

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

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

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

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

14.6.8 Pipet tips -- for automatic pipets.

14.6.9 Coverslips -- for microscope slides.

14.6.10 Lens paper -- for cleaning microscope optics.

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

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

14.6.13 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).

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

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

14.6.16 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).

14.6.17 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.

14.6.18 Tris (hydroxymethyl) aminomethane and hydrogen peroxide (for H_2O_2 spawning method) -- for spawning abalone.

14.6.19 Reference toxicant solutions (see Subsection 14.10.2.4 and see Section 4, Quality Assurance).

14.6.20 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).

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

14.6.22 Dilution water and hypersaline brine -- see Section 7, Dilution Water and Section 14.6.24, Hypersaline Brines. The dilution water should be uncontaminated 1-µm-filtered natural seawater. Hypersaline brine should be prepared from dilution water.

14.6.23 HYPERSALINE BRINES

14.6.23.1 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.

14.6.23.2 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).

14.6.23.3 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.

14.6.23.4 Freeze Preparation of Brine

14.6.23.4.1 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).

14.6.23.4.2 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%.

14.6.23.4.3 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.

14.6.23.5 Heat Preparation of Brine

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

14.6.23.5.1 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.

14.6.23.5.2 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.

14.6.23.5.3 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.

14.6.23.5.4 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.

14.6.23.6 Artificial Sea Salts

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

14.6.23.7 Dilution Water Preparation from Brine

14.6.23.7.1 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.

14.6.23.7.2 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.

14.6.23.8 Test Solution Salinity Adjustment

14.6.23.8.1 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.

14.6.23.8.2 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).

14.6.23.8.3 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:

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

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

14.6.23.9 Preparing Test Solutions

14.6.23.9.1 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-L mark with dilution water, stopper it, and shake to mix. Distribute equal volumes into the replicate test chambers.

14.6.23.9.2 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.

14.6.23.10 Brine Controls

14.6.23.10.1 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, 16.6.23.8.3) 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.

14.6.24 TEST ORGANISMS

14.6.24.1 The test organisms used for this test are red abalone, *Haliotis rufescens*. This large gastropod mollusc is harvested commercially in southern California and supports a popular recreational fishery throughout the state. It consumes a variety of seaweeds and small incidental organisms, and is an important food source for sea otters, lobsters, and octopods (Hines and Pearse 1892). Abalone are "broadcast" spawners that reproduce by equivalent.ejecting large numbers of gametes into the water column, where fertilization takes place externally. Free-swimming larvae hatch as trochophores, then undergo torsion while passing through a veliger stage. Abalone larvae do not feed during their one to three weeks in the plankton, but exist on energy stored in the yolk sack, supplemented perhaps by the uptake of dissolved amino acids. Once larvae come into contact with suitable substrate, they metamorphose and begin to consume benthic algae using a rasp-like tongue (the radula). Red abalone become reproductive after about two years at a length of about 7 cm, and can live for at least 25 years, growing to 30 cm in length. Refer to Hahn (1989) for a review of abalone life history and culture to Martin et al. (1977), Morse et al (1979) and Hunt and Anderson (1989 and 1993) for previous toxicity studies.

14.6.24.2 Species Identification

14.6.24.2.1 Broodstock should be positively identified to species. Epipodal characteristics provide the best means of identification. All California haliotids have a lacey epipodial fringe, except for the red and black abalone, which have smooth, lobed epipodia. The red abalone can be distinguished from the black by shell coloration and by the number of respiratory pores in the shell (reds have 3 to 4, blacks have 5 to 8). For further information on abalone taxonomy consult Owen et al. (1971), and Morris et al. (1980).

14.6.24.3 Obtaining Broodstock

14.6.24.3.1 Mature red abalone broodstock can be collected from rocky substrates from the intertidal to depths exceeding 30 meters. They are found most commonly in crevices in areas where there is an abundance of macroalgae. State collection permits are usually required for collecting abalone. Collection of

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.

abalone is regulated by California law. Collectors must obtain a scientific collectors permit from the California Department of Fish and Game and observe any regulations regarding collection, transfer, and maintenance of abalone broodstock.

14.6.24.3.2 While abalone captured in the wild can be induced to spawn, those grown or conditioned in the laboratory have been more dependable. Commercial mariculture facilities in California produce large numbers of abalone, and distribution systems exist to supply live spawners to a number of market areas. In any case, broodstock should be obtained from sources free of contamination by toxic substances to avoid genetic or physiological preadaptation to pollutants.

14.6.24.3.3 Abalone broodstock can be transported for short time periods from the field or supply facility in clean covered plastic buckets filled with seawater. Use compressed air, or battery powered pumps to supply aeration. Compressed oxygen is not recommended because bubbled oxygen may induce unintended spawning (Morse et al., 1977). Maintain water temperatures within 3EC of the temperature at the collecting site. Four abalone in a 15-liter bucket should remain healthy for up to four hours under these conditions.

14.6.24.3.4 Abalone can be transported for up to 30 hours in sealed, oxygen-filled plastic bags containing moist (seawater) polyfoam sponges (Hahn, 1989). Cut the polyfoam into sections (about 20 X 40 cm) and allow them to soak in clean seawater for a few minutes. New sponges should be leached in seawater for at least 24 hours. Rinse the sponges in fresh seawater and wring them out well. Place the polyfoam inside double plastic trash bags, then place the abalone on the moist foam. It is important that there is no standing water in the bags. Put the abalone bags into an ice chest (10 to 15 liter), fill the bags with pure oxygen, squeeze the bags to purge out all the air, then refill with oxygen (approximately three liters of oxygen gas will support eight abalone). Seal the bags (air-tight) with a tie or rubber band. Wrap two small (one-liter) blue ice blocks in sections of newspaper (about 15 pages thick) for insulation, and place the wrapped blue ice in a sealed plastic bag in the chest on top of the abalone bags. Fill any remaining space with packing and seal the box for shipping. Avoid transporting the ice chest in temperatures below freezing or above 30EC.

14.6.24.4 Broodstock Culture and Handling

14.6.24.4.1 At the testing facility, place the abalone in aerated tanks with flowing seawater (1 to 2 liter/min). With high water quality, water flow, and aeration, abalone 8 to 10 cm long can be kept at a density of one per liter of tank space or one per 100 cm^2 of tank surface area, whichever provides the lower density. Density should be cut to a maximum of 0.5 per liter in recirculating systems and to a maximum of 0.25 per liter in static tanks. Tanks should be covered for shade and to prevent escape. Drain and rinse culture tanks twice weekly to prevent build-up of detritus. Remove any dead abalone immediately, and drain and scrub its tank.

14.6.24.4.2 Ideal maintenance temperature is 15 ± 1EC, the toxicity test temperature (see also Leighton, 1974). If broodstock are to be held for longer than 5 days at the testing facility, feed broodstock with blades of the giant kelp, *Macrocystis*. Feed to slight excess; large amounts of uneaten algae will foul culture water. If *Macrocystis* is unavailable, other brown algae (*Nereocystis*, *Egregia*, *Eisenia*) or any fleshy red algae can be substituted (Hahn, 1989).

14.6.24.4.3 Recirculating tanks should be equipped with biological or activated carbon filtration systems and oyster shell beds to maintain water quality. Measure the ammonia content of static or recirculating seawater daily to monitor the effectiveness of the filtration system. Un-ionized ammonia concentrations should not exceed 20 µg/liter and total ammonia concentrations should not exceed 1.0 mg/liter. Supply constant aeration and temperature control. Add only a few blades of algal food at each cleaning to prevent its accumulation and decay.

14.6.24.4.4 When handling abalone, use a rounded, dull-bladed stainless-steel butter knife, abalone iron, or plastic putty knife to release the animal's grip on the substrate. Gently slide the flat dull blade under the foot at the posterior end near the beginning of the shell whorl, and slide it under about two-thirds of the foot. Apply constant pressure to keep the front edge of the blade against the substrate and not up into the foot. Quickly and gently lift the foot off the substrate. A smooth deliberate motion is more effective and less damaging than repeated prying.

14.6.24.4.5 Assess the reproductive condition of the broodstock by examining the gonads, located under the right posterior edge of the shell. An abalone placed upside down on a flat surface will soon relax and begin moving the foot trying to right itself. Take advantage of this movement and use the dull blade to bend the foot away from the gonad area for inspection. The female ovary is jade green, the male testes are cream-colored. When the gonad fully envelopes the dark blue-gray conical digestive gland and is bulky along its entire length, the abalone is ready for spawning (Hahn, 1989). Ripe (recrudescent) spawners have a distinct color difference between the gray digestive gland and the green or cream-colored gonad. Less developed gonads appear gray (in females) or brown (in males).

14.6.24.4.6 Abalone 7 to 10 cm in shell length are recommended in broodstock. They are easier to handle than larger ones, and can be spawned more often (approximately every four months under suitable culture conditions; Ault, 1985). Though spawning fewer eggs than larger abalone, 10 cm abalone will produce over 100,000 eggs at a time (Ault, 1985). Twenty to thirty-five thousand eggs are needed for a single toxicant test, depending on test design. For further information of red abalone culture, see Ebert and Houk (1984) or Hahn (1989).

14.6.24.5 Culture Materials

14.6.24.5.1 See Section 4, Quality Assurance Section for a discussion of suitable materials to be used in laboratory culture of abalone. Be sure all new materials are properly leached in seawater before use. After use, all culture materials should be washed in soap and water, then rinsed with seawater before reuse.

14.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

14.8 CALIBRATION AND STANDARDIZATION

14.8.1 See Section 4, Quality Assurance.

14.9 QUALITY CONTROL

14.9.1 See Section 4, Quality Assurance.

14.10 **TEST PROCEDURES**

14.10.1 TEST DESIGN

14.10.1.1 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.

14.10.1.2 Effluent concentrations are expressed as percent effluent.

14.10.2 TEST SOLUTIONS

14.10.2.1 Receiving waters

14.10.2.1.1 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 would require approximately 1 L of sample per test.

14.10.2.2 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 66% at 34‰ salinity.

14.10.2.2.2 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%).

14.10.2.2.3 The volume in each test chamber is 200 mL.

14.10.2.2.4 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.

14.10.2.3 Dilution Water

14.10.2.3.1 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.

14.10.2.4 Reference Toxicant Test

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

14.10.2.4.2 The preferred reference toxicant for red abalone is zinc sulfate ($ZnSO_4 \circ H_2O$). 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 10,000 µg/L zinc stock solution by adding 0.0440 g of zinc sulfate ($ZnSO_4 \circ H_2O$) to one liter of reagent water in a polyethylene volumetric flask. Alternatively, certified standard solutions can be ordered from commercial companies.

14.10.2.4.3 Reference toxicant solutions should be five replicates each of 0 (control), 10, 18, 32, and 56, and 100 µg/L total zinc. Prepare one liter of each concentration by adding 0,

1.0, 1.8, 3.2, 5.6, and 10.0 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.

14.10.2.4.4 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 34 ± 2‰.

14.10.3 COLLECTION OF GAMETES FOR THE TEST

14.10.3.1 Spawning Induction

14.10.3.1.1 Note: Before beginning the spawning induction process, be sure that test solutions will be mixed, sampled, and temperature equilibrated in time to receive the newly fertilized eggs. Spawning induction generally takes about three hours, but if embryos are ready before test solutions are at the proper temperature, the delay may allow embryos to develop past the one-cell stage before transfer to the toxicant. Transfer can then damage the embryos, leading to unacceptable test results.

14.10.3.1.2 Culture work (spawning, etc.) and toxicant work should be done in separate laboratory rooms, and care should be taken to avoid contaminating organisms prior to testing.

14.10.3.1.3 Ripe abalone can be induced to spawn by stimulating the synthesis of prostoglandin-endoperoxide in the reproductive tissues (Morse et al., 1977). This can be done in two ways: addition of hydrogen peroxide to seawater buffered with Tris (Morse et al., 1977), or irradiation of seawater with ultraviolet light (Kikuchi and Uki, 1974). The first method is preferable for small laboratories because it avoids the cost and maintenance requirements of a UV system. If a UV system is available, this method may be preferable because it is simple, does not use chemicals that could accidentally harm larvae, and is considered to be less likely to force gametes from unripe adults.

14.10.3.1.4 If brood stock are shipped to the laboratory by a supplier, it is important to allow two days or more for laboratory acclimation before spawning induction; this should

increase the probability of achieving a successful spawn of viable gametes. Always bring brood stock up to acclimation temperature slowly to avoid premature spawning.

14.10.3.2 Hydrogen Peroxide Method

14.10.3.2.1 Select four ripe male abalone and four ripe females. Clean their shells of any loose debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the chambers with flowing or recirculating (1 liter/minute) 20-µm-filtered seawater (15EC), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1EC for 24 hours without food to eliminate wastes. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with 6 liters of 1 µm-filtered seawater. If abalone have been kept in larger aquaria, put them in the buckets at this time. Check the abalone from time to time to make sure they remain underwater. Add air stones to the buckets and keep them aerated until spawning begins.

14.10.3.2.1 Dissolve 12.1 g of Tris into 50 mL of reagent water. When the Tris has dissolved completely, mix the hydrogen peroxide (H_2O_2) solution in a separate flask by pouring 10 mL of fresh* refrigerated H_2O_2 (30%) into 40 mL of refrigerated reagent water (1:5 dilution). Pour 25 mL of Tris solution and 25 mL of H_2O_2 solution into each of the spawning buckets (male and female). Stir well to mix; the final concentration in the spawning buckets will be approximately 6 mM Tris (pH = 9.1) and 5 m H_2O_2. Allow the abalone to remain in contact with the chemicals for 2.5 hours at 15 ± 1EC. The chemical reaction is temperature dependent (three hours of contact with H_2O_2 would be necessary at 11EC). Temperatures higher than 15EC are not recommended for spawning. Maintain constant aeration. Since females often begin spawning after the males, it may be useful to induce male spawning 15-30 minutes later, however egg quality should not be compromised if females spawn first (See 14.10.3.3.2 below).

*Note: Hydrogen peroxide loses potency over time. Purchase reagent or certified grade H_2O_2 in small containers (100

mL). Store unopened containers for no more than one year, and discard open containers after one month. Mark the purchase date and opening date on all containers, and keep all containers refrigerated.

14.10.3.2.3 After 2.5 hours, empty the spawning buckets, rinse them well, and refill them to the top with fresh dilution water seawater at the same temperature (15 ± 1EC). Keep the containers clean by siphoning away mucus and debris. Maintain constant aeration until spawning begins, then remove the air stones. The abalone begin spawning about three hours after the introduction of the chemicals (at 15 ± 1EC). Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.2.4 If spawning begins before the chemicals have been removed, drain the buckets immediately, discarding any gametes. Rinse the buckets thoroughly and refill with clean, dilution water seawater (15 ± 1EC). Use only the gametes subsequently spawned in clean water for testing.

14.10.3.3 UV Irradiation Method

14.10.3.3.1 Select four ripe male abalone and four ripe females. Clean their shells of any debris. Place the males in one clean polyethylene bucket and the females in another. Cover the buckets with a tight fitting perforated lid, supply the containers with flowing or recirculating (1 liter/minute) 20-µm-filtered seawater (15 ± 1EC), and leave the animals without food for 24 to 48 hours to acclimate and eliminate wastes. If flowing seawater is unavailable, keep the spawners in larger (>30 liter) aquaria with aeration at 15 ± 1EC for 24 hours. Three hours prior to the desired spawning time, drain the buckets, wipe and rinse out mucus and debris, and refill with just enough water to cover the abalone (which should all be placed in the bottom of the bucket). Begin slowly filling the buckets with dilution water seawater (15 ± 1EC) that has passed through the UV sterilization unit. Flow rates to each of the buckets should be 150 mL/min. A low total flow rate (300 mL/minute) in the UV unit is necessary to permit sufficient seawater irradiation. (The sterilization unit should be cleaned and the UV bulb replaced at least once annually). Place the buckets in a water bath at 15 ± 1EC to counter the temperature increase caused by the slow

passage of the water past the UV lamp. Check the containers periodically, and keep them clean by siphoning out any debris. After three hours (± about 1/2 hour), abalone should begin spawning by ejecting clouds of gametes into the water. Eggs are dark green and are visible individually to the naked eye, sperm appear as white clouds emanating from the respiratory pores.

14.10.3.3.2 Note: If past experience or other factors indicate difficulties in achieving synchronous spawning, it may be helpful to induce a second group of females about an hour after the first. This will increase the chances of providing fresh eggs (less than one hour old) for fertilization if males spawn late (see below). Senescence of sperm is seldom a problem because males continue spawning over a longer period of time.

14.10.3.4 Pooling Gametes

14.10.3.4.1 Although it is not necessary, it is preferable to have more than one abalone of each sex spawn. To increase the probability of multiple spawners without risking senescence of the gametes, allow one-half hour after the first individual of the second sex begins to spawn before initiating fertilization. For example, if males spawn first, wait one-half hour after the first female spawns before fertilizing eggs. In most cases this will provide time for more than one of each sex to spawn. More important than multiple spawning, however, is avoiding delay of fertilization. Eggs should be fertilized within one hour of release (Uki and Kikuchi 1974). All sperm should be pooled, and all eggs should be pooled prior to fertilization. This can be accomplished by gentle swirling within the spawning buckets. Note: Take care to avoid contaminating eggs with sperm prior to the intended fertilization time. It is important that development is synchronous among all test embryos.

14.10.3.5 Fertilization

14.10.3.5.1 As the females spawn, allow the eggs to settle to the bottom. If necessary, gently stir to evenly distribute the eggs. Siphon out and discard any eggs that appear clumped together. Eggs are ready to transfer to a third (fertilization) bucket when either: (1) one-half hour has passed since the first individual of the second sex has spawned (2) multiple individuals of each sex have spawned, or 3) there are too many eggs on the

bottom of the bucket to allow evenly distributed eggs to avoid each other. Slowly siphon eggs into a third clean polyethylene bucket containing one or two liters of dilution water seawater (15 ± 1EC). Siphon carefully to avoid damaging the eggs and to avoid collecting any debris from the spawning container. Siphon about 100,000 eggs, enough to make a single even layer on the container bottom. Each egg should be individually distinguishable, and not touching other eggs. If excess eggs are available, siphon them into a second fertilization bucket to be used as a reserve. Keep all containers at 15 ± 1EC. Make sure that water temperatures differ by no more than 1EC when transferring eggs or sperm from one container to another.

14.10.3.5.2 As the males spawn, siphon sperm from directly above the respiratory pore and collect this in a 500 mL flask with filtered seawater. Keep the flask at 15 ± 1EC, and use it as a back-up in case the males stop spawning. If spawning continues renew this reserve every 15 minutes. Usually the males will continue spawning, turning the water in the bucket milky white. As long as the males continue spawning, partially drain and refill the bucket every 15 minutes, replacing old sperm-laden water with fresh seawater (15 ± 1EC). Use the freshest sperm possible for fertilization.

14.10.3.5.3 Make sure eggs are fertilized within one hour of release (Uki and Kikuchi, 1974, see note after Section 14.8.5.2). To fertilize the eggs, collect about 200 mL of sperm-laden water in a small beaker. The sperm concentration in the beaker does not have to be exact, just enough to give a slightly cloudy appearance (approximately 1 to 10 X 10^6 cells/mL in the fertilization bucket). See Hahn (1989) for further information on sperm concentrations and the method for fertilization. Pour the sperm solution into the fertilization bucket containing the clean isolated eggs. Using a hose fitted with a clean glass tube, add dilution water seawater to the fertilization bucket at a low flow rate (<1 liter/min; 15 ± 1EC). Use the water flow to gently roil the eggs to allow them to mix with the sperm and fertilize. When the bucket is about half-full and eggs are evenly mixed, stop the water flow and allow the eggs to settle to the bottom of the bucket (about 15 minutes). Fertilization is then complete.

14.10.3.5.4 Note: Once fertilized eggs have settled to the bottom of the bucket (15 minutes after addition of sperm), the following steps (rinsing, concentrating, and counting the embryos) must proceed without delay to assure that embryos are transferred into the test solutions within about one hour. Embryos must be delivered to the test chambers before the first cell division takes place. (Multicellular embryos are more susceptible to damage in handling, and test endpoint analysis assumes that the first cell division takes place in the toxicant solution).

14.10.3.5.5 After embryos have settled, carefully pour or siphon off the water from above the settled embryos to remove as much of the sperm laden water as possible without losing substantial numbers of embryos. Slowly refill the bucket with dilution water seawater (15 ± 1EC). Allow the embryos to settle, and siphon them into a tall 1000 mL beaker for counting. Siphon at a slow flow rate, and move the siphon along the bottom of the bucket quickly to pick up a large number of embryos in the short amount of time it takes to fill the beaker. Examine a sample of the embryos at 100X magnification. One to one hundred sperm should be visible around the circumference of each embryo, 15 sperm per egg is optimal. If sperm are so dense that the embryos appear fuzzy (>>100 sperm/egg), the abalone may develop abnormally and should not be used.

14.10.4 START OF THE TEST

14.10.4.1 Prior to Beginning the Test

14.10.4.1.1 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).

14.10.4.1.2 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.

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

14.10.4.1.4 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 abalone have been examined at the end of the test.

14.10.4.1.5 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.

14.10.4.1.6 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.

14.10.4.2 Estimation of Embryo Density

14.10.4.2.1 Evenly mix the embryos in the 1000 mL beaker by gentle vertical stirring with a clean perforated plunger. Never allow embryos to settle densely in the bottom of the beaker, and take care not to crush embryos while stirring. Take a sample of the evenly suspended embryos using a 1 mL wide bore graduated pipet. Hold the pipet up to the light and count the individual embryos using a hand counter. Alternatively, empty the contents of the pipet onto a Sedgewick-Rafter slide and count embryos under low magnification on a compound scope. Discard the sampled

embryos after counting. Density of embryos in the beaker should be between 200 and 300 embryos/mL. Dilute if the concentration is too high, let embryos settle and pour off excess water if concentration is too low. Take the mean of five samples from this solution to estimate the number of embryos per milliliter.

14.10.4.3 Delivery of Fertilized Embryos

14.10.4.3.1 Using the estimated embryo density in the 1000 mL beaker, calculate the volume of water that contains 1000 embryos. Remove 1000 embryos (or less for smaller volumes, see Section 14.10.1.3) by drawing the appropriate volume of water from the well-mixed beaker using a 10 mL wide bore pipet. Deliver the embryos into the test chambers directly from the pipet making sure not to touch the pipet to the test solution. Stir the embryo beaker with the plunger before taking aliquots. The temperature of the embryo suspension must be within 1EC of the temperature of the test solution. (As above, all solutions are kept at 15 ± 1EC). Record the volume of water delivered into the test chambers with the embryos. Embryos must be delivered into the test solutions within one hour of fertilization. Immediately after the embryos have been delivered, take a sample from the embryo beaker and examine it under 100X magnification. All embryos should still be in the one-cell stage; record any observations to the contrary on the data sheet.

14.10.4.4 Incubation

14.10.4.4.1 Incubate test organisms for 48 hours in the test chambers at 15 ± 1EC under low lighting (approximately 10 $\mu E/m^2/s$) with 16L:8D photoperiod. Fertilized embryos become trochophore larvae, hatch, and develop into veliger larvae in the test solutions during the exposure period.

14.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

14.10.5.1 The light quality and intensity should be at ambient laboratory conditions. Light intensity should be 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a 16 h light and 8 h dark cycle.

14.10.5.2 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.

14.10.5.3 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.

14.10.5.4 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.

14.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

14.10.6.1 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.

14.10.7 OBSERVATIONS DURING THE TEST

14.10.7.1 Routine Chemical and Physical Observations

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

14.10.7.1.2 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.

14.10.7.1.3 Record all the measurements on the data sheet.

14.10.8 TERMINATION OF THE TEST

14.10.8.1 Ending the Test

14.10.8.1.1 Record the time the test is terminated.

14.10.8.1.2 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.

14.10.8.2 Sample Preservation

14.10.8.2.1 After 48 hours exposure, the abalone larvae are fixed in formalin or glutaraldehyde. The two methods for sample preservation are described. Be sure that samples for physicochemical measurements have been taken before further processing of test solutions.

14.10.8.2.2 At the end of the 48-hour incubation period, remove each test chamber, swirl the solution to suspend all the larvae, and pour the entire contents through a 37 µm-mesh screen. The test solution is discarded and the larvae are retained on the screen. Using streams of filtered seawater from a squeeze bottle, rinse the larvae from the screen through a funnel into 25 mL screw cap vials. Be careful not to hit the larvae directly with the streams of water; rough handling during transfer may cause fragmentation of the larvae, making counting more difficult and less accurate. Add enough buffered formalin to preserve larvae in a 5% solution (some laboratories have successfully preserved larvae with lower formalin concentrations. Under-preserved larvae disintegrate quickly, however, and whole tests may have to be rejected if larvae have not been adequately fixed). Addition of formalin is more accurate if the vials are premarked with lines showing the volume of sample and the volume

of formalin to be added. Alternatively, a 0.05% final glutaraldehyde solution may be substituted. Larvae should be counted within two weeks.

14.10.8.2.3 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.

14.10.8.3 Counting

14.10.8.3.1 To count the larvae using a standard compound microscope, pipet all the larvae from the bottom of the preservation vial onto a Sedgewick-Rafter counting cell. Examine 100 larvae from each vial under 100X magnification. To best characterize the sample and to avoid bias, select groups of larvae one field of vision at a time, moving to the next field without looking through the lens. Be careful to work across the slide in one direction to avoid recounting the same areas. Count the number of normal and abnormal larvae using hand counters. The percent normal larvae is calculated as the number normal divided by the total number counted. After counting, use a funnel to return the larvae to the vial for future reference.

14.10.8.4 Endpoint

14.10.8.4.1 Examine the shape of the larval shell to distinguish normal from abnormal larvae. Count veliger larvae as normal if they have smoothly curved larval shells that are striated with calcareous deposits and are somewhat opaque. It is common for normal larvae to have a slight curved indentation near the leading edge of the shell. A single indentation is this area is counted as normal.

14.10.8.4.2 Larvae with both multiple indentations and an obvious lack of calcification (i.e. clear appearance in at least part of the shell) are counted as abnormal. The combination of these two features indicates inhibition of a biological process (lack of calcification) and actual damage to the organism (indentations) allowed by the thin shell. Refer to the accompanying photographs (Figure 1) for classification of

marginally deformed larvae. The following types of larvae are also counted as abnormal: (1) larvae that have arrested development (from one cell through trochophore stage), (2) larvae with obvious severe deformations, (3) larvae with broken shells, (4) larval shells separated from the rest of the animal, and (5) larvae found remaining in the egg membrane (however, take care to distinguish these from larvae that may have come in contact with loose egg cases). Record all counts and the test chamber number on the data sheet.

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

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

14.12 **ACCEPTABILITY OF TEST RESULTS**

14.12.1 Test results are acceptable only if all the following requirements are met:

(1) the mean larval normality must be at least 80% in the controls.

(2) the response from 56 µg/L zinc treatment must be significantly different from the control response.

(3) the minimum significant difference (%MSD) is <20% relative to the control for the reference toxicant.

14.13 **DATA ANALYSIS**

14.13.1 GENERAL

14.13.1.1 Tabulate and summarize the data. Calculate the proportion of larvae with normally developed shells for each replicate. A sample set of test data is listed in Table 4.

14.13.1.2 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.

FIGURE 1. 48-HOUR-OLD ABALONE VELIGER LARVAE

Figures 1A -D Provided by John Hunt, Institute of Marine Sciences. Photocopied from:

"Marine Bioassay Project Procedures Manual of October, 1990." California State Water Resources Control Board.

The following three pages show 12 photographs of 48-hour-old abalone veliger larvae from effluent toxicity tests. All larvae were taken from intermediate effluent concentrations and were chosen to represent "borderline" cases (i.e. larvae that were slightly affected and are therefore, difficult to categorize as normal or abnormal). In most cases, larvae from lower and higher effluent concentrations are more easily categorized than those shown here; in the lower concentrations they are obviously without shell abnormalities and in the higher concentrations they are severely deformed. These photographs are presented as a visual reference to help standardize test analysis and eliminate bias in the interpretation of marginally deformed larvae. All larvae on the left-hand side of these pages were counted as normal, all larvae on the right-hand side were counted as abnormal.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR, *HALIOTIS RUFESCENS*, LARVAL DEVELOPMENT 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 light
5. Light intensity:	10 µE/m^2/s (Ambient laboratory levels)
6. Photoperiod:	16 h light, 8 h darkness
7. Test chamber size:	600 mL
8. Test solution volume:	200 mL/replicate
9. Larvae density per chamber:	5-10 per mL
10. No. replicate chambers per concentration:	5
11. Dilution water:	Uncontaminated 1-µm-filtered natural seawater or hypersaline brine plus reagent water
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. Endpoint:	Normal shell development

16. Test acceptability criteria:	$80% normal shell development in the controls; must have statistical significant effect at 56 µg/L zinc; must achieve a %MSD of <20%
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

14.13.1.3 The endpoints of toxicity tests using the red abalone are based on the reduction in proportion of normal shell development. 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.

14.13.2 EXAMPLE OF ANALYSIS OF RED ABALONE, *HALIOTUS RUFESCENS*, LARVAL DEVELOPMENT DATA

14.13.2.1 Formal statistical analysis of the larval development is outlined in Figure 2. The response used in the analysis is the proportion of larvae with normally developed shells 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 shell 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.

TABLE 4. DATA FROM RED ABALONE, *HALIOTUS RUFESCENS*, DEVELOPMENT TEST

Effluent Concentration (%)	Replicate	No. Larvae Counted	Number Normal	Proportion Normal
Brine	A	100	100	1.00
Control	B	100	98	0.98
	C	100	100	1.00
	D	100	99	0.99
	E	100	99	0.99
Dilution	A	100	99	0.99
Control	B	100	99	0.99
	C	100	99	0.99
	D	100	100	1.00
0.56	A	100	99	0.99
	B	100	99	0.99
	C	100	98	0.98
	D	100	100	1.00
	E	100	100	1.00
1.00	A	100	99	0.99
	B	100	100	1.00
	C	100	99	0.99
	D	100	99	0.99
	E	100	100	1.00
1.80	A	100	99	0.99
	B	100	99	0.99
	C	100	99	0.99
	D	100	98	0.98
	E	100	97	0.97
3.20	A	100	39	0.39
	B	100	57	0.57
	C	100	61	0.61
	D	100	65	0.65
	E	100	80	0.80
5.60	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00
10.00	A	100	0	0.00
	B	100	0	0.00
	C	100	0	0.00
	D	100	0	0.00
	E	100	0	0.00

14.13.2.2 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.

14.13.2.3 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.

14.13.2.4 Comparison of Brine and Dilution Controls

14.13.2.4.1 This example uses toxicity data from a red abalone, *Haliotus rufescens*, larval development test performed with effluent. The response of interest is the proportion of larvae with normally developed shells, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. Because the example test was run using both brine and dilution controls, the two controls must first be tested for significant differences in the normal shell development proportions. The raw and transformed data, means and variances of the transformed observations for the two controls are listed in Table 5.

14.13.2.4.2 Tests for Normality

14.13.2.4.2.1 In the two sample situation, the distributional assumption is that each sample comes from a normally distributed population. Thus in comparing the brine and dilution controls, the data for each concentration must be separately checked for normality. When the two response groups are tested separately, it is not necessary to center the data.

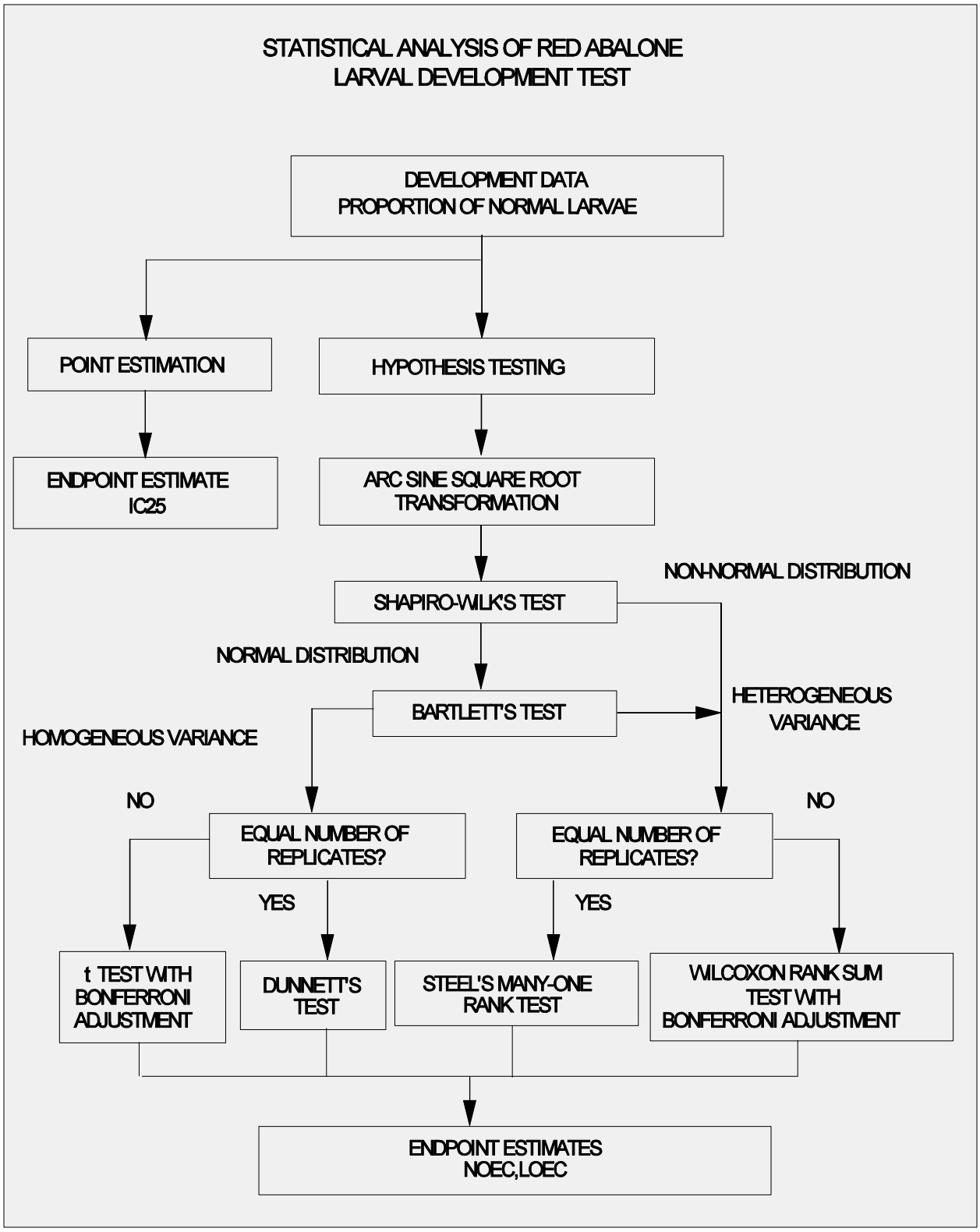

Figure 2. Flowchart for statistical analysis of red abalone, *Haliotus rufescens*, development data.

TABLE 5. RED ABALONE, *HALIOTUS RUFESCENS*, LARVAL DEVELOPMENT DATA FROM BRINE AND DILUTION CONTROLS

	Replicate	Brine Control	Dilution Control
RAW	A	1.00	0.99
	B	0.98	0.99
	C	1.00	0.99
	D	0.99	1.00
	E	0.99	
ARC SINE SQUARE ROOT TRANSFORMED	A	1.521	1.471
	B	1.429	1.471
	C	1.521	1.471
	D	1.471	1.521
	E	1.471	
Mean ($\bar{X}_i$)		1.483	1.484
S_i^2		0.00152	0.000625
i		1	2

14.13.2.4.2.2 Calculate the denominator, D, of the statistic for each control group:

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

Where: X_i = the ith centered observation

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

n = the total number of centered observations

14.13.2.4.2.3 For the brine control data,

n = 5

$$\bar{X} = \frac{1}{5}(7.413) = 1.483$$

$$D = 0.00609$$

For the dilution control data,

$$n = 4$$

$$\bar{X} = \frac{1}{4}(5.934) = 1.484$$

$$D = 0.00191$$

14.13.2.4.2.4 Order the observations for each control group from smallest to largest

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

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for the two groups in this example are listed in Table 6.

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

Brine Control		Dilution Control	
i	$X^{(i)}$	i	$X^{(i)}$
1	1.429	1	1.471
2	1.471	2	1.471
3	1.471	3	1.471
4	1.521	4	1.521
5	1.521		

14.13.2.4.2.5 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 datasets in this example, n = 5 and k = 2 for the brine control group, and n = 4 and k = 2 for the dilution control group. The a_i values are listed in Table 7.

TABLE 7. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$
Brine Control Group		
1	0.6646	0.092	$X^{(5)} - X^{(1)}$
2	0.2413	0.050	$X^{(4)} - X^{(3)}$
Dilution Control Group		
1	0.6872	0.050	$X^{(4)} - X^{(1)}$
2	0.1667	0.000	$X^{(3)} - X^{(2)}$

14.13.2.4.2.6 Compute the test statistic, W, for each group 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 7. For the data in the brine example:

$$W = \frac{1}{0.00609} (0.07321)^2 = 0.880$$

For the data in the dilution example:

$$W = \frac{1}{0.00191} (0.03436)^2 = 0.618$$

14.13.2.4.2.7 The decision rule for this test is to compare W as

calculated in Subsection 2.6 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 the brine control, the critical value at a significance level of 0.01 and n = 5 observations is 0.686. Since W = 0.880 is greater than the critical value, conclude that the brine control data are normally distributed. For the data in the dilution control, the critical value at a significance level of 0.01 and n = 4 observations is 0.687. Since W = 0.618 is less than the critical value, conclude that the dilution control data are not normally distributed.

14.13.2.4.2.8 Since the dilution control data does not meet the normality assumption, the Wilcoxon Rank Sum Test will be used to compare the responses in the two control groups.

14.13.2.4.3 Wilcoxon Rank Sum Test

14.13.2.4.3.1 To perform the Wilcoxon Rank Sum test, combine the data from the two groups and arrange in order from smallest to largest. Assign the rands (1, 2, ..., 9) 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. A table of the ranks is given in Table 8.

TABLE 8. ASSIGNING RANKS TO THE BRINE AND DILUTION CONTROLS FOR THE WILCOXON RANK SUM TEST

Rank	Transformed Proportion Normal	Control Group
1	1.429	Brine
4	1.471	Brine
4	1.471	Brine
4	1.471	Dilution
4	1.471	Dilution
4	1.471	Dilution
8	1.521	Brine
8	1.521	Dilution
8	1.521	Dilution

14.13.2.4.3.2 The ranks are then summed for both of the control groups. For this data, the sum of the ranks in the brine control group is 25 and the sum of the ranks in the dilution control group is 20.

14.13.2.4.3.3 For this situation, we wish to determine if the proportions of normally developed larvae in the two control groups are significantly different. To do this, compare the rank sum of the group with the smaller sample size with some "minimum" or critical rank sum, at or below which the devlopment in the controls would be considered significantly different. At a significance level of 0.05, the minimum rank sum in a test with five replicates in one group and and four replicates in the other is 11 (See Snedecor and Cochran, 1980).

14.13.2.4.3.4 The dilution control sample size is smaller than the sample size of the brine control group so its rank sum is compared to the critical value. Since its rank sum of 20 is greater than the critical value of 11, conclude that the development proportions for the two control groups are not significantly different.

14.13.2.5 Example of Analysis of Larval Development Data

14.13.2.5.1 Since the responses in the two control groups are not significantly different, only the dilution control group will be used in the analysis of the shell development responses for the effluent concentrations. As above, each replicate must first 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 effluent concentration and dilution control are listed in Table 9. The data are plotted in Figure 3. Since there is 100% abnormality in all replicates for the 5.6% and 10.0% concentrations, they are not included in the statistical analysis and are considered qualitative abnormality effects.

14.13.2.6 Test for Normality

14.13.2.6.1 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 10.

14.13.2.6.2 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

14.13.2.6.3 For this set of data, n = 24

$\bar{X} = \frac{1}{24}$ (-0.004) = 0.000

D = 0.1127

14.13.2.6.4 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 11.

14.13.2.6.5 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 12.

14.13.2.5.6 Compute the test statistic, W, as follows:

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

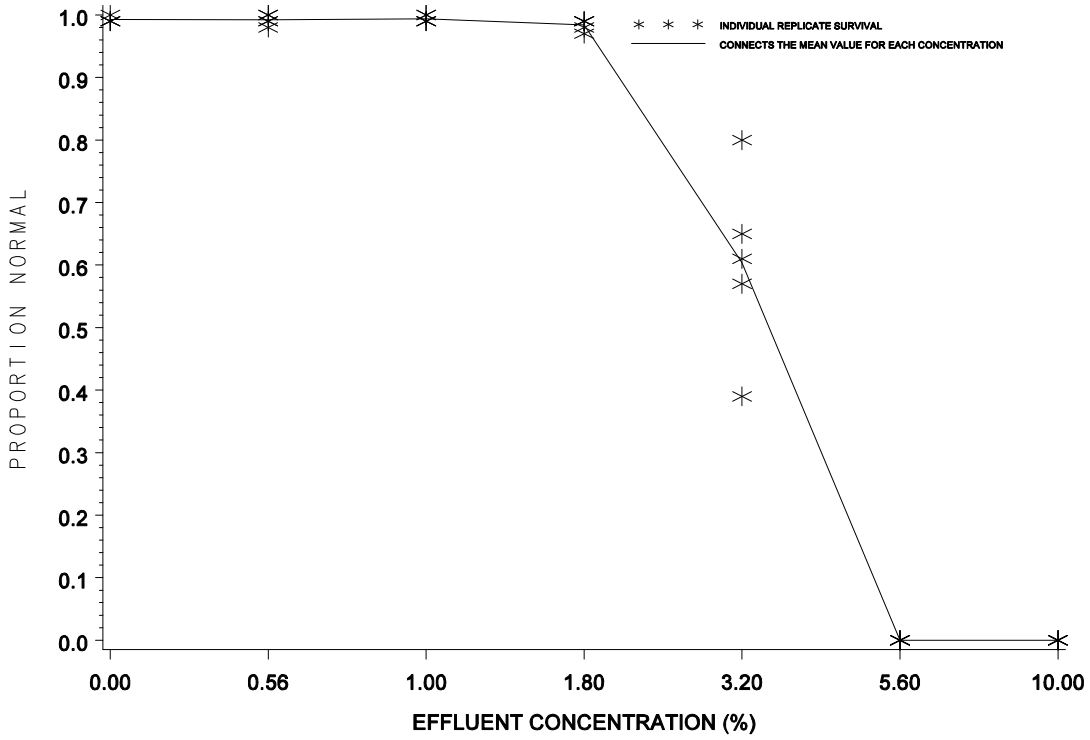

Figure 3. Plot of mean proportion of normally developed red abalone, *Haliotus rufescens* larvae.

TABLE 9. RED ABALONE, *HALIOTUS RUFESCENS*, SHELL DEVELOPMENT DATA

		Effluent Concentration (%)						
	Dilution	Control	0.56	1.00	1.80	3.20	5.6	10.0
	Replicate							
RAW	A	0.99	0.99	0.99	0.99	0.39	0	0
	B	0.99	0.99	1.00	0.99	0.57	0	0
	C	0.99	0.98	0.99	0.99	0.61	0	0
	D	1.00	1.00	0.99	0.98	0.65	0	0
	E		1.00	1.00	0.97	0.80	0	0
ARC SINE SQUARE ROOT TRANSFORMED	A	1.471	1.471	1.471	1.471	0.674	-	-
	B	1.471	1.471	1.521	1.471	0.856	-	-
	C	1.471	1.429	1.471	1.471	0.896	-	-
	D	1.521	1.521	1.471	1.429	0.938	-	-
	E		1.521	1.521	1.397	1.107	-	-
Mean ($\bar{x}_i$)		1.484	1.483	1.491	1.448	0.894	-	-
S_i^2		0.000625	0.001523	0.000750	0.001137	0.024288	-	-
i		1	2	3	4	5	6	7

TABLE 10. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

	Effluent Concentration (%)				
Replicate	Control	0.56	1.00	1.80	3.20
A	-0.013	-0.012	-0.020	0.023	-0.220
B	-0.013	-0.012	0.030	0.023	-0.038
C	-0.013	-0.054	-0.020	0.023	0.002
D	0.037	0.038	-0.020	-0.019	0.044
E		0.038	0.030	-0.051	0.213

TABLE 11. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.220	13	-0.012
2	-0.054	14	0.002
3	-0.051	15	0.023
4	-0.038	16	0.023
5	-0.020	17	0.023
6	-0.020	18	0.030
7	-0.020	19	0.030
8	-0.019	20	0.037
9	-0.013	21	0.038
10	-0.013	22	0.038

TABLE 12. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4493	0.433	$X^{(24)} - X^{(1)}$
2	0.3098	0.098	$X^{(23)} - X^{(2)}$
3	0.2554	0.089	$X^{(22)} - X^{(3)}$
4	0.2145	0.076	$X^{(21)} - X^{(4)}$
5	0.1807	0.057	$X^{(20)} - X^{(5)}$
6	0.1512	0.050	$X^{(19)} - X^{(6)}$
7	0.1245	0.050	$X^{(18)} - X^{(7)}$
8	0.0997	0.042	$X^{(17)} - X^{(8)}$
9	0.0764	0.036	$X^{(16)} - X^{(9)}$
10	0.0539	0.036	$X^{(15)} - X^{(10)}$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 12. For the data in this example:

$$W = \frac{1}{0.1127} (0.2974)^2 = 0.7848$$

14.13.2.5.7 The decision rule for this test is to compare W as calculated in 14.13.2.5.6 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 = 0.7848 is less than the critical value, conclude that the data are not normally distributed.

14.13.2.5.8 Since the data do not meet the assumption of normality, the Wilcoxon Rank Sum Test with the Bonferroni Adjustment will be used to analyze the shell development data.

14.13.2.6 Wilcoxon Rank Sum Test with the Bonferroni Adjustment

14.13.2.6.1 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, ... , 9) 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.

14.13.2.6.2 An example of assigning ranks to the combined data for the control and 0.56% concentration is given in Table 13. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 14. The ranks are then summed for each concentration level, as shown in Table 15.

14.13.2.6.3 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 four concentrations (excluding the control), four control replicates and five concentration replicates is 15 (See Table 5, Appendix F).

TABLE 13. ASSIGNING RANKS TO THE CONTROL AND 0.56% CONCENTRATION LEVEL FOR THE WILCOXON RANK SUM TEST WITH THE BONFERRONI ADJUSTMENT

Rank	Transformed Proportion Normal	Concentration
1	1.429	0.56 %
4	1.471	0.56 %
4	1.471	0.56 %
4	1.471	Control
4	1.471	Control
4	1.471	Control
8	1.521	0.56 %
8	1.521	0.56 %
8	1.521	Control

TABLE 14. TABLE OF RANKS[1]

Replicate	Control	Effluent Concentration (%) 0.56	1.00	1.80	3.20
1	1.471(4,3.5,5.5,7)	1.471(4)	1.471(3.5)	1.471(5.5)	0.674(1)
2	1.471(4,3.5,5.5,7)	1.471(4)	1.521(8)	1.471(5.5)	0.856(2)
3	1.471(4,3.5,5.5,7)	1.429(1)	1.471(3.5)	1.471(5.5)	0.896(3)
4	1.521(8,8,9,9)	1.521(8)	1.471(3.5)	1.429(2)	0.938(4)
5		1.521(8)	1.521(8)	1.397(1)	1.107(5)

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

TABLE 15. RANK SUMS

Concentration (% Effluent)	Rank Sum
0.56	25.0
1.00	26.5
1.80	19.5
3.20	15.0

14.13.2.6.4 Since the rank sum for the 3.20% concentration level is equal to the critical value, the proportion normal in that concentration is considered significantly less than that in the control. Since no other rank sum is less than or equal to the critical value, no other concentration has a significantly lower proportion normal than the control. Hence, the NOEC and the LOEC are 1.80% and 3.20%, respectively.

14.13.2.7 Calculation of the ICp

14.13.2.7.1 The shell development data in Table 4 are utilized in this example. As can be seen from Table 4 and Figure 4, the observed means are not 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, the means are smoothed prior to calculating the IC. In the following discussion, the observed means are represented by $\bar{x}_i$ and the smoothed means by M_i.

14.13.2.7.2 Starting with the control mean, $\bar{x}_1 = 0.993$ and $\bar{x}_2 = 0.992$, we see that $\bar{x}_1 > \bar{x}_2$. Set $M_1 = \bar{x}_1$. Comparing $\bar{x}_2$ to $\bar{x}_3$, $\bar{x}_2 < \bar{x}_3$.

14.13.2.7.3 Calculate the smoothed means:

$$M_2 = M_3 = (\bar{x}_2 + \bar{x}_3)/2 = 0.993$$

14.13.2.7.4 Since $\bar{x}_7 = 0 < \bar{x}_6 = 0 < \bar{x}_5 = 0.604 < \bar{x}_4 = 0.984 < \bar{x}_3 = 0.993$, set $M_3 = 0.993$, $M_4 = 0.984$, $M_5 = 0.604$, $M_6 = 0$, and set $M_7 = 0$.

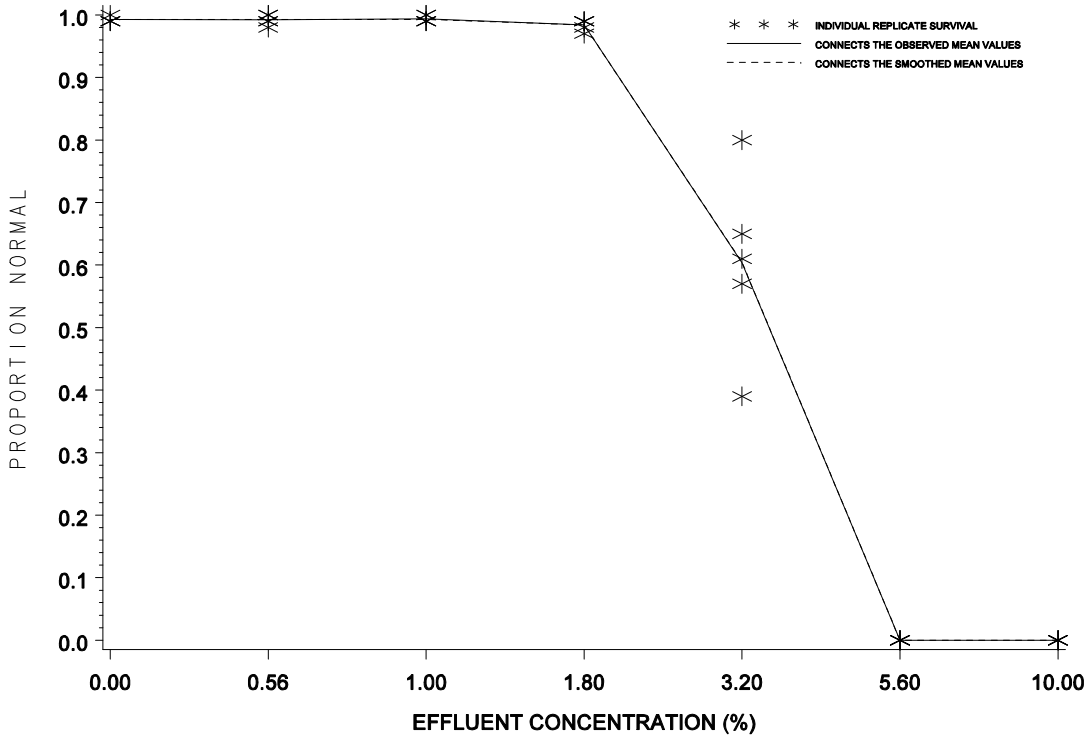

Figure 4. Plot of raw data, observed means, and smoothed means for red abalone, *Haliotus rufescens*, development data from Tables 4 and 16.

TABLE 16. RED ABALONE, *HALIOTUS RUFESCENS*, MEAN SHELL DEVELOPMENT RESPONSE AFTER SMOOTHING

Effluent Conc. (%)	i	Response Means, $\bar{Y}_i$ (proportion)	Smoothed Means, M_i (proportion)
Control	1	0.993	0.993
0.56	2	0.992	0.993
1.00	3	0.994	0.993
1.80	4	0.984	0.984
3.20	5	0.604	0.604
5.60	6	0.000	0.000
10.00	7	0.000	0.000

14.13.2.7.5 Table 16 contains the response means and smoothed means and Figure 4 gives a plot of the smoothed response curve.

14.13.2.7.6 An IC25 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.745, where $M_1(1-p/100) = 0.993(1-25/100)$. Examining the means and their associated concentrations (Table 16), the response, 0.745, is bracketed by $C_4 = 1.80\%$ effluent and $C_5 = 3.20\%$ effluent.

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

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

$$IC25 = 1.8 + [0.993(1 - 25/100) - 0.984] \left(\frac{(3.2 - 1.8)}{(0.604 - 0.984)}\right)$$

$$= 2.68\%.$$

14.13.2.7.8 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 2.6818%. The empirical 95.0% confidence interval for the true

mean was 2.5000% to 3.1262 %. The computer program output for the IC25 for this data set is shown in Figure 5.

14.14 **PRECISION AND ACCURACY**

14.14.1 PRECISION

14.14.1.1 Single-Laboratory Precision

14.14.1.1.1 Data on the single laboratory precision of the *Haliotis rufescens* larval development method using zinc sulfate are shown in Table 17. Zinc concentrations were 18, 32, 56, and 100 µg/L. All tests were conducted at the Marine Pollution Studies Laboratory. There was good agreement among test EC50s, with a coefficient of variation of 8%.

14.14.1.2 Multi-laboratory Precision

14.14.1.2.1 The multi-laboratory data indicate a similar level of test precision Table 18. Data are presented for four interlaboratory trials in which either two or three laboratories tested both split effluent samples and reference toxicants. The mean coefficient of variation between EC50 values from different laboratories was 15%.

14.14.2 ACCURACY

14.14.2.1 The accuracy of toxicity tests cannot be determined.

```
Conc. ID        1    2    3    4    5    6    7
-----------------------------------------------
Conc. Tested    0  .56    1  1.8  3.2  5.6   10
-----------------------------------------------
Response  1   .99  .99  .99  .99  .39    0    0
Response  2   .99  .99 1.00  .99  .57    0    0
Response  3   .99  .98  .99  .99  .61    0    0
Response  4  1.00 1.00  .99  .98  .65    0    0
Response  5       1.00 1.00  .97  .80    0    0
-----------------------------------------------
*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Effluent
Test Start Date:    Test Ending Date:
Test Species: Red Abalone
Test Duration:        48 hours
DATA FILE: abalone.icp
OUTPUT FILE: abalone.i25
-------------------------------------------------------------------
Conc.     Number     Concentration     Response      Std.       Pooled
 ID     Replicates                %       Means       Dev.   Response Means
-------------------------------------------------------------------------
  1          4             0.000          0.993      0.005      0.993
  2          5             0.560          0.992      0.008      0.993
  3          5             1.000          0.994      0.005      0.993
  4          5             1.800          0.984      0.009      0.984
  5          5             3.200          0.604      0.148      0.604
  6          5             5.600          0.000      0.000      0.000
  7          5            10.000          0.000      0.000      0.000
-------------------------------------------------------------------------
The Linear Interpolation Estimate:     2.6818   Entered P Value: 25
-------------------------------------------------------------------------
Number of Resamplings:   80
The Bootstrap Estimates Mean:   2.7085 Standard Deviation:     0.1510
Original Confidence Limits:   Lower:     2.5000 Upper:     3.1262
Expanded Confidence Limits:   Lower:     2.4091 Upper:     3.3484
Resampling time in Seconds:     0.27  Random_Seed: -770872716
```

Figure 5. ICPIN program output for the IC25.

TABLE 17. SINGLE LABORATORY PRECISION DATA FOR THE RED ABALONE, *HALIOTIS RUFESCENS* LARVAL DEVELOPMENT TEST WITH ZINC (ZN FG/L) SULFATE AS A REFERENCE TOXICANT

Test Date	NOEC (µg/L)	EC50 (µg/L)
March 1990	32	42[1]
May 1990	32	39[1]
January 1991	18	34[1]
February 1991	18	40[2]
Mean		38.4
SD		3.0
CV (%)		7.8

1 Source: Hunt et al., 1991
2 Source: Anderson et al., 1994

TABLE 18. MULTI-LABORATORY PRECISION OF THE RED ABALONE, *HALIOTIS RUFESCENS* LARVAL DEVELOPMENT TEST PERFORMED WITH ZINC (ZN FG/L) SULFATE AND EFFLUENT (%) AS THE TOXICANTS

Test Date	Toxicant	Lab	NOEC	EC50	CV
March 1990	Effluent	A	>3.2%	nc	
March 1990	Effluent	B	>3.2%	nc	
March 1990	Effluent	C	0.32%	1.83	nc
March 1990	Zinc	A	32	41	
March 1990	Zinc	B	18	28	
March 1990	Zinc	C	18	31	20%
May 1990	Effluent	A	3.2%	4.7	
May 1990	Effluent	D	1.8%	3.5	
May 1990	Effluent	C	3.2%	3.8	16%
May 1990	Zinc	A	32	39	
May 1990	Zinc	D	32	46	
May 1990	Zinc	C	32	37	12%
January 1991	Effluent	A	<0.56%	1.5	
January 1991	Effluent	C	1.25%	1.8	13%
January 1991	Zinc	A	18	34	
January 1991	Zinc	C	32	48	24%
January 1991	Effluent	A	1.0%	2.7	
January 1991	Effluent	C	1.8%	2.8	3.0%

Mean Interlaboratory CV = 15% Interlaboratory CV based on 6 tests for which CV values could be calculated. Source: Hunt et al., 1991.

nc = indicates that the CV could not be calculated because only one laboratory observed a 50% effect and calculated an EC50.

APPENDIX I. RED ABALONE 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 zinc reference toxicant stock solution (10,000 µg/L) by adding 0.0440 g of zinc sulfate ($ZnSO_4 \circ 7H_2O$) to 1 liter of reagent water.

D. Prepare zinc reference toxicant solution of 0 (control) 10, 18, 32, 56 and 100 µg/L by adding 0, 1.0 1.8, 3.2, 5.6 and 10.0 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. Obtain test organisms and hold or condition as necessary for spawning.

B. Induce four male and four female abalone to spawn using either H_2O_2 and Tris or UV irradiated seawater (300 mL/min flow rate through the UV unit). All solutions should be maintained at 15 ± 1EC.

C. Siphon eggs into a fertilization bucket. Add 200 mL of sperm-laden water to fertilize the eggs. Wash the fertilized eggs at least twice by slowly decanting and refilling the chamber with fresh filtered seawater. Temperatures should vary by no more than 1EC between waters used in mixing and refilling.

D. Suspend the embryos evenly in a 1000 mL beaker and count five samples in a 1 mL pipet to estimate embryo density.

E. Pipet 1000 fertilized embryos into each 200 mL test chamber. Be sure temperatures in the embryo beaker and the solutions are at 15 ± 1EC. Incubate for 48 h. For smaller-sized chambers, use proportionately fewer embryos.

F. At the end of the 48 h period, pour the entire test solution with larvae through a 37 µm-meshed screen. Wash larvae from the screen into 25 mL vials. Add buffered formalin to preserve the larvae in a 5% solution or glutaraldehyde for a 0.05% solution. Cap the flask and invert gently to mix.

G. Pipet a sample from each vial onto a Sedgwick-Rafter counting slide and count 100 larvae. Return the larvae to the vials for future reference.

H. Count the number of normal larvae for each replicate and divide by the total counted.

I. Analyze the data.

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

Salinity Adjustment Worksheet for Abalone

Date Sampled: Batch:
Date Adjusted: Region:

$\frac{VS \quad (TS-SS)}{(SB - TS)}$ SS = Salinity of Sample VB = Volume of Brine
VS = Volume of Sample SB = Salinity of Brine
TS = Target Salinity (34±2%)

VDW = VBL - VBS VDW = Volume of Dilution Water (Adjusted to 34±2%)
VBL = Largest Volume of Brine added to adjust salinity
VBS = Volume of Brine added to each Sample

Total Volume = VB added + VDW added
(Total volume should be the same for all samples)

Site Code (ID Org #) or concentration	Initial Salinity	TS	Vol. of Brine	Vol.Dil. Water	Total Volume	Final Salinity	Precision and Accuracy for Refractometer
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
		34±2					
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		34±2					
		34±2					
		34±2					
		34±2					

Initials: Double Checked:

Data Sheet for Mollusc Larval Development Toxicity Test

Test Start Date: Start Time: Mollusc Species:

Test End Date: End Time: Collection/Arrival Date:

Reference Toxicant:Reference Toxicant: Broodstock Source:

Sample Source:

Sample Type: Effluent Ref Tox Solid Elutriate Pore Water WaterSample Type: Effluent Ref Tox Solid Elutriate Pore Water Water

Test Cont. #	Station Code or Concentration	Sample ID #	After 48 hours		Notes
			Normal Larvae	Abnormal Larvae	
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
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34					
35					

Computer Data StorageComputer Data Storage Disk:
File:

Notes