DEPARTMENT OF
ECOLOGY
State of Washington

Biological Testing Methods 80-12

For the Designation of Dangerous Waste

Part A: Static Acute Fish Toxicity Test

Part B: Acute Oral Rat Toxicity Test

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Hazardous Waste and Toxics Reduction Program
Washington State Department of Ecology
Olympia, Washington

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Introduction

The Washington State Department of Ecology (Ecology) developed the acute fish toxicity test (Method 80-12) to determine if a waste meets the definition of dangerous waste in the *Dangerous Waste Regulations*, Chapter 173-303 WAC. Appendix A contains a modified test procedure for conducting neutralized fish toxicity tests.

Method 80-12 provides a simple, low cost method for testing the toxicity of a waste against the requirements of WAC 173-303. This method determines if the sample waste LC_{50} is significantly less than or equal to the regulatory threshold of 100 mg/L dangerous waste (DW), 10 mg/L extremely hazardous waste (EHW), or 10 mg/L special waste. The LC_{50}, for this method's purposes, is the median lethal concentration of waste that kills 50 percent of the test fish within 96 hours.

If the toxicity of a waste is unknown, the waste must be tested for dangerous waste designation using Method 80-12. The waste concentrations of 100 mg/L and 10 mg/L were selected to correspond with the definitions of dangerous waste and extremely hazardous waste, respectively. State-only special waste uses a regulatory threshold of 10 mg/L.

Waste designated by Method 80-12 must be regulated and managed as specified in WAC 173-303, and must be sampled according to procedures in WAC 173-303-110, Sampling and Testing Methods.

Any person whose waste fails the acute fish toxicity test, and who believes that mortality resulted from effects other than acute toxicity (e.g., disease or physical stress), may petition Ecology to exempt the waste from designation. They may also petition Ecology to modify the test procedure. Any person who believes that test failure resulted **only** because of pH may choose to demonstrate this by performing a neutralized fish bioassay per Appendix A. The purpose of this demonstration would be to revise waste designation as provided in WAC 173-303-910(5). Any pH wastes containing hazardous constituents with inherent toxicity (e.g., ammonia, cyanide) will not qualify for designation changes. If additional toxicity information is needed to further characterize a waste, a toxicity identification evaluation (TIE) procedure may be useful (EPA, 1991a). Petitions must be submitted per WAC 173-303-910.

Health and Safety

Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management, and includes:

- Appointment of a laboratory health and safety officer with the responsibility and authority to develop and maintain a safety program.

- Preparation of a formal, written, health and safety plan, which is provided to each laboratory staff member.
- Ongoing training program on laboratory safety.
- Regularly scheduled, documented, safety inspections.

Toxicity testing may involve significant risks to personal safety and health. Personnel conducting toxicity tests should protect themselves by taking all safety precautions necessary to prevent bodily injury. Observe procedures to prevent inhalation or skin absorption of wastes. Each laboratory should be equipped with all necessary safety equipment prior to conducting toxicity tests.

Personal safety gear, such as rubber aprons, lab coats, respirators, gloves, safety glasses, and safety shoes, should be used by personnel. Each laboratory should have safety equipment such as first aid kits, fire extinguishers, fire alarms, and fire blankets.

Because the chemical composition of waste is usually poorly known, consider them potential health hazards. Minimize exposure to waste samples. Fume and canopy hoods over test areas must be used whenever necessary. Strong acids and volatile organic solvents employed in glassware cleaning must be used in a fume hood or with an exhaust canopy over the work area.

The following recommended laboratory procedures will promote a safe working environment and minimize health risks. This list is by no means exhaustive. Ecology encourages testing laboratories to implement these, and other, recommendations promoting the health and safety of laboratory personnel.

- Testing industrial wastes should be performed in compliance with accepted rules pertaining to the handling of hazardous materials. Ecology recommends that personnel conducting toxicity tests not work alone.
- Wastes should be considered potential health hazards and exposure to them should be minimized.
- Immediately wash any body part that may have come in contact with wastes. Although a water rinse often suffices, note that some wastes may be activated with water.
- Adequately label all containers to indicate contents.
- Good housekeeping habits contribute to safety and reliable results.
- Use electrical equipment approved by Underwriters Laboratories. Ground-fault interrupters should be installed in all "wet" labs where electrical equipment is used.
- Staff training in basic first aid and cardio-pulmonary resuscitation (CPR) is strongly recommended.
- All personnel should receive tetanus immunizations. The following are optional and dependent on the type of waste anticipated: hepatitis B (if suspected contact with human bodily fluids, including blood); hepatitis A, typhoid fever, and polio (if suspected contact with human feces or urine). Polio may be transmitted from infant immunizations through fecal material. Please err on the side of caution. If your laboratory has any questions, contact the Washington Department of Labor and Industries or similar agency in your state.

- Wastes generated during toxicity testing must be handled properly and safely disposed. Each testing facility will have its own waste disposal requirements based on local, state, and federal rules and regulations. All persons responsible for, or otherwise involved in, performing testing activities must know, understand, and comply with these rules and regulations. Local fire officials should be notified of any potentially hazardous conditions.

For further guidance on safe laboratory practices, consult EPA (1986), EPA (2002), and Walters and Jameson (1984). Also see Appendix B for Good Laboratory Procedure Standards.

Methods and Materials

Holding Facilities

Facilities should include tanks for holding/acclimating test fish and a constant-temperature room or recirculating water bath for the test chambers. Holding tanks must be operated on a flow-through basis; this may include either once-through or recirculating systems with at least 50 percent renewal. Holding, acclimation, and dilution-water tanks and headboxes should be equipped for temperature control and aeration. Air used for aeration must be free of oil and fumes; filters to remove oil and water may be used. Test fish should be shielded from disturbances during holding, acclimation, and testing. The test facility should be well ventilated and free of fumes.

Fish should be quarantined at least 7 days when first brought into a facility. Avoid crowding to maintain test fish in good condition during holding and acclimation. The dissolved oxygen (DO) concentration must be maintained between 60 percent and 100 percent saturation; gentle aeration may be used if necessary. Aeration drives off the ammonia from generated biological waste.

A photoperiod of 16 hours of light/8 hours of darkness should be provided during the test. Preferably, though not required, light intensity should be raised gradually over a 15 minute period at the beginning of the photoperiod. Gradually lower the light intensity over the same period of time at the end of the photoperiod. Use a dimmer switch or other suitable device to make this easier. The light intensity should be in the range of natural outdoor light or ambient laboratory illumination (about 10-20 uE/m^2/s or 50-100 ft-c). Avoid high intensity and extreme lighting conditions.

Whether fabricated in the lab or purchased commercially, tanks should not be constructed of materials, or have surface coatings, which will leach or dissolve into aqueous solutions. Tanks and other equipment that contact stock solutions or test solutions should be chosen to minimize sorption of toxicants from water. Use glass, stainless steel, and fluoroplastics whenever possible to minimize leaching, dissolution, and sorption. Concrete and rigid (unplasticized) plastics may be used for holding and acclimation tanks and in the water supply system. Cast iron pipe may be used in freshwater supply systems, but colloidal iron will be added to the dilution water and strainers will be needed to remove rust particles. Natural rubber, copper, brass, galvanized metal, solder, and lead should not come in contact with dilution water, stock solutions, or test solutions before or during exposure of test fish.

Test Chambers

Test chambers should have a minimum solution depth of 15 cm. Chambers filled to within the top 15 cm should be covered to prevent test fish from jumping out. Test chambers commonly used in static tests for fish are wide-mouth glass jars or aquaria, 3.8 L (1 gal) or 19.0 L (5 gal). Special glass or stainless steel test chambers can be constructed to accommodate test fish requiring particular physical conditions (large surface-area-to-volume ratio).

Test chambers may be constructed of 3-mm (0.125 in), double strength, or 6-mm (0.25 in) tempered plate glass held together with clear silicone adhesive. Silicone adhesive absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. As little of the adhesive as possible should be in contact with water; extra beads of adhesive inside the containers should be removed. Stainless steel (No. 304 or No. 316) can be used in the construction of test chambers, but should be of welded, **not soldered**, construction.

Commercially available test chambers meeting the above requirements may be used. Disposable, non-toxic plastic (e.g., polypropylene) liners may be used. Ecology **highly recommends** laboratories using disposable liners perform several rounds of in-house testing to determine that the liners are truly non-toxic before using in dangerous waste designation testing.

Cleaning and Disinfection

Clean test chambers before use. Wash new test chambers with ammonia-based detergent suitable for biological laboratories, then rinse in the following sequence with:

- Clean water.
- Acid, such as 5 percent concentrated nitric acid.
- Copious clean water.
- Reagent grade acetone.
- Copious clean water.

At every test termination, chambers should be cleaned in this sequence:

- Emptied.
- Immediately rinsed with clean water.
- Cleaned using appropriate procedures for removing the toxicant (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds.
- Rinsed with copious clean water.

Acid is useful for removing mineral deposits and 200 mg hypochlorite/L for one hour is useful for removing organic matter and for disinfection. A solution containing 200 mg hypochlorite/L is conveniently made by adding 6 mL of liquid household chlorine bleach to 1 L of water. **Acid and hypochlorite should not be used together because hazardous chlorine fumes may be produced.** Tanks may also be sterilized with an iodophor. Commercially prepared iodophors using polyvinylpyrrolidone-iodine (PVP-I) as the active ingredient, are available as Wescodine, Betadine, Argentyne, or other product names.

For disinfection of equipment such as fish tanks, prepare a 50 mg/L solution of titrateable iodine by diluting a commercially available product. The equipment should be immersed in or washed with

this solution for at least 10 minutes followed by copious rinsing with clean water. Iodophors and hypo-chlorites are acutely toxic to most aquatic organisms. Hypochlorite can be removed by flushing with water or by reacting it with an equivalent concentration of sodium thiosulfate or sodium sulfite. After a tank is washed with hypochlorite and rinsed, air-dry the equipment overnight to allow dissipation of residual chlorine.

Dilution Water

An adequate supply of quality dilution water must be available. Acceptable test dilution water allows healthy fish to survive for the duration of acclimation and testing without showing stress, such as discoloration or unusual behavior. A better criterion for acceptable dilution water is test fish surviving and growing satisfactorily in it. Monthly hardness, alkalinity, and specific conductance ranges must be within less than 10 percent of their respective averages. The monthly pH range must be less than 0.8 units to produce consistent quality dilution water.

Ideally, non-chlorinated dilution water is preferred, but may not be realistic. Dechlorination using activated carbon filters or other methods may be used to dechlorinate dilution water as thoroughly as possible. Chlorine may also be removed using an activated carbon filter then treating with a sodium thiosulfate or sodium sulfite drip. Sodium thiosulfate is better for dechlorinating water than sodium sulfite and may be more reliable than activated carbon for removing chloramines.

Metals can usually be removed with chelating agents. Municipal water supplies often contain unacceptably high concentrations of copper, lead, zinc, and fluoride; buildings may contain copper pipes and lead solder. Toxicity tests can be adversely affected by the use of dilution water containing these materials.

The dilution water should be intensively aerated using air stones, surface aerators, or screen tubes before introducing the toxicant. Adequate aeration will bring the concentration of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and volatiles concentration. The dilution water dissolved oxygen should be between 90 - 100 percent saturation. Rinse test chambers with dilution water just before use.

Dilution water hardness can affect the outcome of the test. Moderately hard dilution water must be used in this method and should not be less than 40 mg/L $CaCO_3$. Low hardness/alkalinity may render test organisms more sensitive to some toxicants. Use reagent-grade chemicals to adjust hardness and alkalinity. Table 1 is for guidance purposes.

Table 1. Preparation of Dilution Water Using Reagent Grade Chemicals[a]

Water Type	$NaHCO_3$ mg/L[b]	$CaSO_4 \bullet 2H_2O$ mg/L[b]	$MgSO_4$ mg/L[b]	KCl mg/L[b]	pH[c]	Total Resulting Hardness[d]	Total Resulting Alkalinity[d]
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70

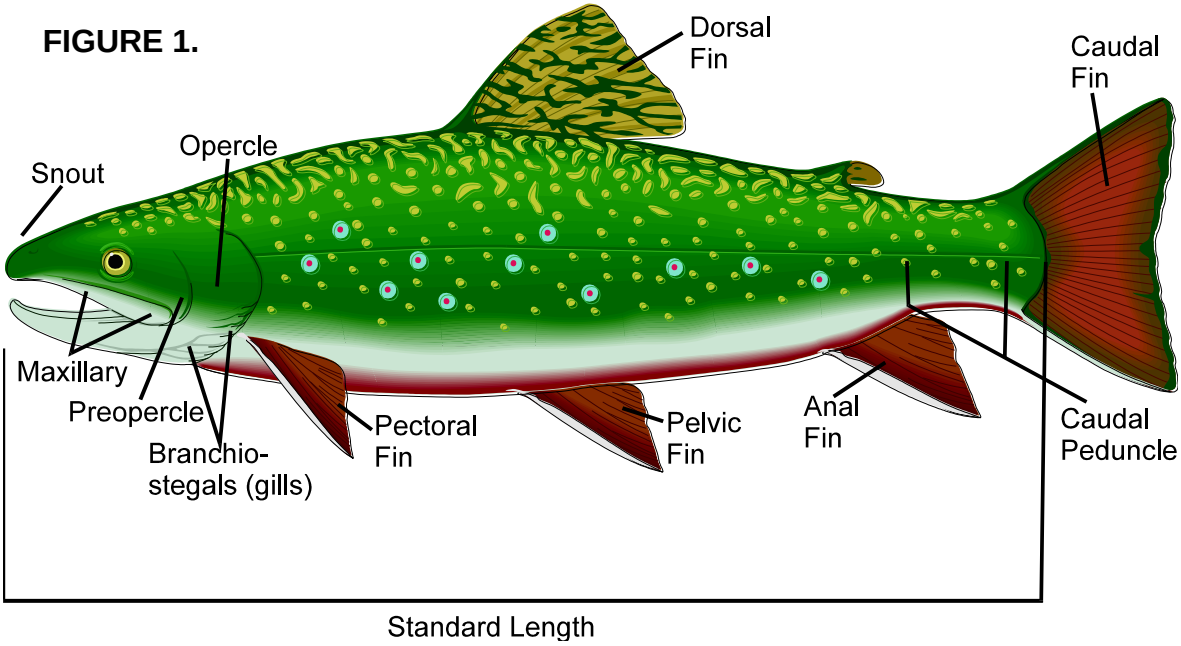
[a] Adapted from EPA (2002)
[b] Add reagent grade chemicals to distilled or deionized water.
[c] Approximate equilibrium pH after aerating 24 hours.
[d] Expressed as mg/L $CaCO_3$.

Test Organisms

Use one of the following species in the static fish toxicity test (all species must be verified): Coho salmon, *Oncorhynchus kisutch*; rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*); and brook trout, *Salvelinus fontinalis*. The test temperature for all species is 12 ± 1 °C.

All fish used in a test should be of the same age and from the same source. Organisms of the same species from different sources may produce different test results. Usual sources of freshwater fish are private, state, and federal hatcheries.

FIGURE 1.



Test fish should be between 30 and 90 days from swim-up and as uniform in size as possible. Circumstances may arise where this range cannot be met; some variation in age is acceptable, provided the variation is no more than four or five days. In any single test, all fish should be from the same batch. The standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish (Figure 1).
All test fish should be obtained from a hatchery that has been certified disease-free for the following diseases; bacterial kidney disease (*Renibacterium salmoninarum*); Costia (*Ichythobodo*); bacterial gill disease (*Myxobactriea sp.*); and Furunculosis (*Aeromonas salmonicida*). **If fish are diseased, destroy the entire lot immediately.** Make all efforts to ensure test fish are disease-free prior to initiation of toxicity test. Options include:

- Selecting fish from a source (hatchery) which will certify that all fish are disease-free.
- Discriminately selecting test fish from a reputable source or on the observations and judgment of a knowledgeable individual.
- Treating all fish that arrive on-site using treatments commonly used by sources (hatcheries) which produce certified disease-free fish.

Between use of different test fish groups, holding and acclimation tanks should be sterilized according to the procedures in **Cleaning and Disinfection**.

Care and Handling

Minimize unnecessary stress to test fish. To avoid unnecessary stress, do not subject fish to rapid changes in temperature or water quality. Generally, aquatic organisms should not be subjected to more than a 3°C change in water temperature in any 12 hour period. Maintain test fish in dilution water at test temperature for at least the last 48 hours before they are placed in test chambers.

Handle test-fish as little as possible. If necessary, handle them as gently, carefully, and quickly as possible. Discard fish that touch dry surfaces, are dropped, or injured during handling. Dip nets are best for handling fish and are commercially available or made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting, or similar material. Equipment used to handle aquatic organisms should be sterilized between uses by autoclaving or treating with an iodophor, 200 mg hypochlorite/L, or 30 percent formalin plus 1 percent benzalkonium chloride for at least 1 hour. Sterilize or wash hands before **and** after handling fish.

Holding

After collection or transportation, quarantine and acclimate fish to laboratory conditions for a minimum of 7 days before used in tests. Hold fish in uncontaminated, aerated water of constant temperature and quality in a flow-through system with a minimum flow rate of five volume exchanges per day (ASTM, 2006a). When possible, hold fish in dilution water and as close to test temperature as possible. During long holding periods, however, it is generally easier and safer to hold fish at temperatures lower than 12°C as the metabolic rate and the number and severity of disease outbreaks are reduced. Recommended fish loading for holding tanks is provided for various water temperatures, fish size, and species, in Appendix D.

If stock fish have 5 percent or greater mortality between 24 hours after arrival on-site and the end of the 7 day acclimation period, the entire lot should not be used for testing. If at any time during the holding period there is an abnormal number of mortalities, do not use the fish for testing.

Stress Indicators

Test fish should be carefully observed daily during holding and acclimation for signs of disease, stress, physical damage, abnormal behavior, and mortality. Dead, injured, and abnormal individuals must be discarded. Visually examine the behavior and external appearance of the fish. If the stressor can be determined, remove stressor and reacclimatize for 7 days. The following physical characteristics indicate stress:

- Darkened color (normal is silver/gray)
- Emaciation
- Erratic swimming
- Excessive mucus production
- External parasites
- Not eating
- Flashing
- Flipping
- Fungus/Fin Rot
- Gasping at surface
- Hemorrhaging
- Hyperventilation

Sample Preparation

Sample Reporting Requirements

All samples must be extracted using the method described below. Solid samples must be reduced in size prior to extraction unless the sample meets the particle size criteria described below. Sample analysis must occur with 45 days of sample collection.

Ensure all phases of the sample are homogenous. Stirring may be required if the sample is not homogenous. Samples may be extracted using a rotary agitation apparatus. The rotary agitation apparatus must be capable of rotating the extraction bottles at 30 ± 2 rpm. The sample is allowed to extract for 18 $\pm$ 2 hours at 23 $\pm$ 2^{o}C. The extraction water is the stock dilution water used in the toxicity test.

Particle Size Reduction

Prior to conducting the extraction, determine the need for reducing the sample particle size. Particle reduction is required unless the solid has a surface area per gram of material equal to or greater than 3.1 cm^2, or is smaller than 1 cm in its narrowest dimension (i.e., is capable of passing through a 9.5 mm (0.375 inch) standard sieve).

If the surface area is smaller or the particle size larger than described above, prepare the solid portion of the waste for extraction by crushing, cutting, or grinding the waste to a surface area or particle-size.

Note: Surface area criteria are meant for filamentous (e.g., paper, cloth, and similar) waste material. Actual measurement of surface area is not required, nor is it recommended. For materials that do not obviously meet the criteria, sample-specific methods would need to be developed and employed to measure the surface area. Such methodology is currently not available.

Rotary Agitation Method

- Weigh out the correct amount of a well-mixed sample and transfer into 500 or 1000 mL extractor bottle.
- Add 200 mL of test dilution water to the extractor bottle.
- Place a Teflon cap liner over the mouth of the extractor bottle and screw on the cap.
- Mix the sample on the rotary agitation apparatus for 18 $\pm$ 2 hours at 23 $\pm$ 2^{o}C, remove the cap and pour the extraction water into the dilution water in the fish test tank. Rinse all loose material from the flask into the fish test tank with a 200 mL aliquot of stock dilution water. Place the bottle into the fish tank so that the bottle is full of water and lying on its side on the tank bottom. Drop the cap liner into the fish test tank.
- Begin the test as described below.
- Extreme care should be exercised throughout the course of the extraction so as not to contaminate the exterior of the extraction flask. For example, sealing sterile flasks in sterile aluminum foil prior to use or copiously rinsing with deionized water, at a minimum, are acceptable methods.

Test Procedure

Replicates and Control

Use a minimum of ten fish in each of three replicate test tanks exposed to each treatment. Additional fish and replicates may be used (e.g., 15 fish in each of four replicates). Replicates must be true replicates with no water connections between the replicate test chambers. Randomize the replicate test chambers and impartially distribute a representative sample of test fish to the chambers.

One randomization method adds to each test chamber no more than 20 percent of the number of test fish to be placed in each test chamber. Repeat this process until each test chamber contains the desired number of test fish. Alternatively, fish can be assigned either by random assignment of one fish to each test chamber, random assignment of a second fish to each chamber, etc., or by total randomization. It is often convenient to assign fish to other containers and then add them to the test chambers.

Every test requires a control consisting of the same dilution water, conditions, number of replicates, procedures, and test species used in the test tanks. None of the toxicants being tested is added to the control.

Test Temperature

The test temperature for all test species must be 12°C. The actual test temperature must not deviate from 12°C by more than ± 1.0°C at any time during the test.
Continuous temperature monitoring in at least one test tank is desirable. Alternatively, the maximum and minimum temperature must be recorded every 24 hours. As a second alternative, temperatures in at least one test tank must be recorded at least every 6 hours.

pH

Do not adjust the pH of the test solution. Any pH adjusting will render the test void. There are provisions for demonstrating that the waste kills fish only because of pH. Appendix A includes a procedure for conducting this type of toxicity test.

The pH may tend to drift during the 96 hour test period. This drift is due, in part, to fish respiration, increasing the CO_2 content of the water, which tends to buffer the solution. Do not adjust the pH when this drift is observed. Record the pH at the time intervals indicated on the data sheet.

Dissolved Oxygen

The saturation value, not concentration, determines the ease of oxygen transport across the gill surface diffusion gradient. The dissolved oxygen in each test chamber should be at least 80 percent (e.g., 8.5 mg/L at 12°C at 250 feet above sea level) of saturation at the beginning of the test. The dissolved oxygen should not fall below 60 percent (e.g., 6.4 mg/L at 12°C at 250 feet above sea level) of saturation during the first 48 hours of the test. It must not fall below 50 percent

saturation during the remainder of the test. The test must be voided if the dissolved oxygen falls below 50 percent saturation during the test and mortality exceeds the pass/fail threshold. Please refer to Appendix D for information on dissolved oxygen saturation and concentration levels at different altitudes.

Test solutions may be gently aerated if dissolved oxygen does not meet the above criteria. Once aeration is initiated, continue throughout test duration. Avoid turbulence; it stresses fish, resuspends fecal matter, and increases volatilization. Because evaporation readily occurs at the surface, efficient aeration can be achieved with minimum turbulence by using an air lift to transfer solution from the bottom to the surface. Aeration should be identical in all test chambers

Loading

Loading in test chambers should not exceed 0.8 g/L. The g/L of test fish (wet weight, blotted dry) to solution in test chambers should not be so high as to affect test results. To determine loading, use same number of fish as per replicate but do not use these fish in the actual test. Lower loadings are recommended if dissolved oxygen does not remain above 60 percent saturation for the first 48 hours of the test and above 50 percent saturation after 48 hours. Limit loading to ensure:

- Concentrations of dissolved oxygen and toxicant are not decreased below acceptable levels.
- Concentrations of metabolic products does not increase above acceptable levels.
- Fish are not stressed due to crowding.

Feeding

Do not feed test fish during acute toxicity tests or for a minimum of 48 hours before the test. If the test fish are fed less than 48 hours before test initiation, fecal matter and uneaten food may decrease the dissolved oxygen concentration and otherwise affect the biological activity of some toxicants.

Duration and Acceptability

Begin the test by placing the flask of waste and leachate in the test chamber on its side. The cap should also be placed in the test chamber.

The flask exterior should be sterile or as clean as possible to avoid introducing additional contamination. "Blank" flasks used in the extraction process should be used for the control chambers.

The test begins when test fish are first exposed to the toxicant and ends 96 hours later. Control test chambers must show 90 percent survival or greater for test acceptance or validity.

Test Observations and Measurements

Measure dissolved oxygen and the pH in all test tanks at the beginning. Repeat these measurements every 24 hours (as long as live fish are present). Also record the dissolved oxygen and pH at the end of the test or at the time the last fish dies in the test tank. Hardness, alkalinity, pH, and conductivity must be measured in the test and control tanks at the start of the test.

Measure the same parameters at the end of the test. Measurement of calcium, magnesium, sodium, potassium, chloride, sulfate, particulate matter, and total organic carbon (TOC) or chemical oxygen demand (COD) is also desirable.

The number of dead or affected fish in each test chamber must be counted every 24 hours after starting the test. More observations are desirable, especially near test initiation. Ecology suggests counting the number of dead or affected fish in each chamber 4, 8, and 24 hours (or similar time increments) after the beginning of the test. Repeat the count twice daily thereafter to the end of the test.

Remove dead fish as soon as they are observed, or once every 24 hours. Do not stress live test fish when determining whether test fish are dead, immobilized, or otherwise affected, and removing dead fish. Movement of test chambers and prodding must be very gentle.

Criteria for Death

Death is the adverse effect most often used in acute toxicity tests. The criteria for death are lack of movement, especially the absence of respiratory movements in fish and lack of reaction to gentle prodding. The loss of equilibrium, defined as the inability to make coordinated movement and maintain a normal upright position, is not considered mortality. The latter responses can be useful in interpretation of test results and should be noted in the comment section of the data-reporting sheet.

Termination

Weights and lengths of test fish may be determined by weighing and measuring and discarding either; (a) a representative group of fish before the test; or (b) the control fish that are alive at the end of the test. The length should be reported as standard length and the weights should be wet weight, blotted dry. Destroy all fish, both control and treatment, at termination of test, after data collection is complete.

Data Analysis

Refer to **Calculation of Results**. The number of mortalities needed to designate a waste is no longer a single number for dangerous waste or extremely hazardous waste. This "threshold" number of mortalities is now a function of the mean and variance in the control group and the mean and variance in the test group and does not utilize a data transformation.

Quality Assurance / Quality Control

Procedures

Reliable laboratory data is critical for making sound environmental decisions. A good quality assurance/quality control (QA/QC) program is essential to ensure data reliability. Quality assurance (QA) is the total integrated program for assuring the reliability of monitoring data. A comprehensive QA program addresses everything affecting data and should address:

- Sample collection and preservation.

- Laboratory services.
- Use and care of instruments, glassware, and chemicals.
- Data management.
- Laboratory personnel.

Quality control (QC) is the routine application of statistically based procedures to evaluate and control the accuracy of results from analytical measurements. A comprehensive QC program includes procedures for estimating and controlling precision and bias, as well as:

- Defines data quality objectives.
- Chooses analytical methods which will meet those objectives.
- Estimates within-lab precision and bias.
- Sets up control charts.
- Participates in inter-lab tests.

Reference Toxicants

Reference toxicants establish the validity of toxicity data. The reference toxicant measures all aspects of toxicity testing including organism quality, presence and effects of stressors other than test compounds, and the quality of analytical techniques. If reference toxicant results (LC_{50}s) differ significantly from previously established results (LC_{50} control charts), a full review of laboratory test conditions and procedures is indicated. Results that are not within two standard deviations of the mean LC_{50}, should be considered "red flags."

Evaluate procedures and test fish with a reference toxicant once a month or, preferably, concurrently with the test. Reference toxicant testing is required for each new batch of fish regardless of whether a test has been run that month on another batch of fish. More frequent testing with a reference toxicant is appropriate when situations occur which may affect test results. For example, a change in dilution or holding water conditions, new equipment or piping in the laboratory, or a change of analysts, all indicate the need for additional use of a reference toxicant.

Various compounds are commonly used as reference toxicants and include phenol, sodium chloride (NaCl), potassium chloride (KCl), zinc chloride ($ZnCl_2$), and zinc sulfate ($ZnSO_4$). This is not an all inclusive list by any means. To ensure nominal concentrations of reference toxicants, concentrations should be measured periodically.

Calculation of Results

The bioassay results are calculated using the statistical method described below; two examples are given. Within a 90 percent confidence limit, the test sample fails the bioassay by exhibiting a median lethal concentration (LC_{50}) less than or equal to (i.e., toxicity greater than or equal to) the regulatory threshold (i.e., 100 or 10 mg/L) when the calculated t statistic is greater than a critical t value.

Approach

The statistical analysis is based on methodology presented by Zar (1984), EPA (2002), and Erickson and McDonald (1995). Because observations on individual fish in the same tank cannot necessarily be considered statistically independent, the experimental unit is the tank and not individual fish. This avoids a statistical error in experimental design referred to as pseudoreplication (Hurlbert, 1984).

The two-sample t test is used. This test assumes both control and test groups are randomly selected from normal populations with equal variances. However, the frequency of samples displaying varying proportions of dead and live fish, as determined in acute lethality bioassays, is described by the binomial distribution. Use of the two-sample t test is robust enough to stand considerable departures from its theoretical assumptions, especially if sample sizes are equal (Zar, 1984). Untransformed sample data have been shown to be normally distributed, in most cases, as assessed by Shapiro-Wilk's test for normality (EPA, 2002).

The type of t test used depends on the homogeneity of variance assumption between control and test samples (Zar, 1984; EPA, 2002). This assumption is evaluated with the variance ratio F test. If variances are equal, the equal variance t test is used. If variances are not equal, a modified t test is used.

Thus, the appropriate t test is used to compare the difference between test and control mortality proportions against a constant, $p_o = 0.5$, corresponding to the LC_{50}. Up to ten percent mortality in the control sample is allowed. *A one-tailed t test is used, since the analysis determines only if the sample LC_{50} is significantly greater than the regulatory threshold.* This objective is consistent with the procedure for waste designation from bioassay data outlined in WAC 173-303-100(5)(c).

Hypothesis

In terms of concentration, the one-tailed null hypothesis (H_o) to be tested is $LC_{50} \leq 100$ mg/L (dangerous waste threshold) or $LC_{50} \leq 10$ mg/L (extremely hazardous waste threshold or special waste threshold). In terms of mortality, each of these hypotheses corresponds to a one-tailed H_o: $(p_{Tm} - p_{Cm}) \geq p_o$, where p_{Tm} and p_{Cm} are the mean proportion of mortalities in the test and control samples, respectively, and $p_o = 0.5$ is the proportion of mortalities at the LC_{50}. An alpha level of 0.10 is used in the t test (i.e., one-tailed 90 percent confidence level), consistent with statistical methodology outlined by EPA (1986). Rejecting H_o demonstrates the sample does not designate as a regulated waste.

Calculation

1. Sample size: The number of replicates in the control and test groups should be equal and should each consist of at least three ($N \geq 3$). The number of fish per replicate tank should be equal across all replicates and should consist of at least ten ($n \geq 10$). In addition, the number of fish per replicate tank should not exceed a biomass loading density of 0.8 g/L.

2. Mortalities: Mortalities are expressed as proportions:
p_C = proportion of mortalities in control replicate.
p_T = proportion of mortalities in test replicate.

The bioassay is invalid if overall control mortalities exceed 10 percent (EPA, 2002).

3. F test: The variance ratio F test is used to identify the appropriate t test. A two-tailed F test is conducted at an alpha level of 0.05 with N - 1 degrees of freedom (df) in both numerator and denominator (Zar, 1984; EPA, 2002):

$F = s_C^2/s_T^2$ or $F = s_T^2/s_C^2$, whichever is larger.

where: F = F statistic
s_C^2 = variance for the control group.
s_T^2 = variance for the test group.

If the calculated F value is less than or equal to the critical F value, the variances are equal and the equal variance t test is used. If the calculated F value is greater than the critical F value, the variances are not equal and the modified t test should be used. *Note that in the case where both variances are zero, the variances are equal. However, when only one variance is zero, the variances are considered statistically unequal.*

4. Equal variance t test: With an equal number of replicates in the control and test groups, the equal variance t test has the following form (Zar, 1984):

$t = (p_{Tm} - p_{Cm} - p_o) / (2s_p^2/N)^{0.5}$ (general equation) or

$t = (p_{Tm} - p_{Cm} - 0.5) / [(s_T^2 + s_C^2)/N]^{0.5}$

where: t = t statistic
p_{Tm} = mean p_T
p_{Cm} = mean p_C
p_o = 0.5 = proportion of mortalities at LC_{50}
s_p^2 = pooled variance = $(s_T^2 + s_C^2)/2$
N = number of replicates in control or test group.

The number of degrees of freedom in an equal variance t test with an equal number of replicates in control and test groups is 2(N - 1). The calculated t statistic is compared to a one-tailed critical t value at an alpha level of 0.10 with 2(N - 1) degrees of freedom (df). When the calculated t value is less than or equal to the critical t value, H_o is rejected, and it is concluded that the waste does not designate as a regulated waste. Note that in the case where $(s_T^2 + s_C^2) = 0$, H_o is rejected when $(p_{Tm} - p_{Cm}) < 0.5$ and H_o is accepted when $(p_{Tm} - p_{Cm}) \geq 0.5$.

5. Modified t test: With an equal number of replicates in the control and test groups, the modified t test for unequal variances has the same form as the equal variance t test above (Zar, 1984). However, the number of degrees of freedom differs and is equal to the following (Zar, 1984):

$df = (N - 1)(s_C^2 + s_T^2)^2 / [(s_C^2)^2 + (s_T^2)^2]$

If the computed degrees of freedom are non-integer, use the next smaller integer. Note that if either $s_C^2 = 0$ or $s_T^2 = 0$, then df = N - 1. The calculated t statistic is compared to a critical t value

with the modified degrees of freedom. The interpretation then follows that described above for the equal variance t test.

6. Critical F and t values: Tables 2 and 3 list critical F and t values, respectively. Appropriate critical values for other degrees of freedom can be found in most statistics books (e.g., Zar, 1984). For number of degrees of freedom, see section numbers 3-5 under 'Calculation'.

Table 2. Critical F Values With Two-Tailed α = 0.05

Denominator df	Numerator df			
	2	3	4	5
2	39.0	39.2	39.2	39.3
3	16.0	15.4	15.1	14.9
4	10.6	9.98	9.60	9.36
5	8.43	7.76	7.39	7.15

Table 3. Critical T Values With One-Tailed α = 0.10

df	t[1]	df	t
1	-3.078	6	-1.440
2	-1.886	7	-1.415
3	-1.638	8	-1.397
4	-1.533	9	-1.383
5	-1.476	10	-1.372

[1] Critical t values are negative, since the interest lies only in whether (p_T - p_C) is significantly smaller than 0.5.

Example 1: Equal Variance t Test

This test consists of two treatments and one control, each in triplicate. Two sets of three replicates of ten fish each are exposed to 10 and 100 mg/L, respectively, of waste using the 80-12 bioassay procedure to determine if the waste sample designates as dangerous waste or extremely hazardous waste. The control group is exposed to dilution water only.

Table 4. Test Results

Replicate (N=3)	Dilution Water Control (n=10)		10 mg/L Sample (n=10)		100 mg/L Sample (n=10)	
	Number Dead	Proportion Dead (p_C)	Number Dead	Proportion Dead (p_T)	Number Dead	Proportion Dead (p_T)
A	1	0.100	1	0.100	4	0.400
B	1	0.100	2	0.200	6	0.600
C	0	0	1	0.100	5	0.500
Mean (p_m)		0.067		0.133		0.500
Variance (s^2)[1]		0.0033		0.0033		0.0100

[1] Variance equation is $s^2=\Sigma(p-p_m)^2/N-1$ where p = p_C or p_T

After 96 hours, control and test tank mortalities are tabulated. Proportions are based on number of fish per replicate (e.g., 1/10 = 0.100). The critical F and t values are found in Tables 2 and 3, respectively.

Table 5. Summary for Equal Variance T Test

	10 mg/L	100 mg/L
Calculated F statistic	0.0033/0.0033 = 1.00	0.0100/0.0033 = 3.03
Critical F df; numerator & denominator each = (N - 1)	2, 2	2, 2
Critical F *(see Table ~~1~~2)*	39.0	39.0
Calculated t[1] statistic	-9.25	-1.01
Critical t df[2] = [2(N-1)]	4	4
Critical t *(see Table ~~2~~3)*	-1.53	-1.53
Does waste designate?	NO	YES
Waste Code	not applicable	WT02

[1] 10 mg/L calculated t = $(0.133-0.067-0.5)/[(0.0033+0.0033)/3]^{0.5}$ & 100 mg/L calculated t = $(0.5-0.067-0.5)/[(0.0033+0.0100)/3]^{0.5}$
[2] 2(N-1) is only used in the equal variance t test

With the appropriate equations, calculate F = 1.00 (10 mg/L) and F = 3.03 (100 mg/L). Because both of the calculated F statistics are less than the critical F value (39.0), the variances are equal and the equal variance t test is performed. The calculated t statistics are -9.25 (10 mg/L) and -1.01 (100 mg/L). Because the 10 mg/L calculated t statistic (-9.25) is less than the critical t value (-1.53), H_o is rejected and the waste does **not** designate as extremely hazardous waste. However, because the 100 mg/L calculated t statistic (-1.01) is greater than the critical t value (-1.53), H_o is **not** rejected and the waste **does** designate as dangerous waste.

Example 2: Modified t Test

This test consists of two treatments and one control, each in quadruplicate. Two sets of four replicates of 15 fish each are exposed to 10 and 100 mg/L, respectively, of waste using the 80-12 bioassay procedure to determine if the waste sample designates as dangerous waste or extremely hazardous waste. The control group is exposed to dilution water only.

Table 6. Test Results

Replicate (N=4)	Dilution Water Control (n=15)		10 mg/L Sample (n=15)		100 mg/L Sample (n=15)	
	Number Dead	Proportion Dead (p_C)	Number Dead	Proportion Dead (p_T)	Number Dead	Proportion Dead (p_T)
A	0	0	4	0.267	9	0.600
B	0	0	6	0.400	8	0.533
C	0	0	3	0.200	10	0.667
D	0	0	0	0	6	0.400
Mean (p_m)		0		0.217		0.550
Variance (s^2)[1]		0		0.0278		0.0130

[1] Variance equation is $s^2=\Sigma(p-p_m)^2/N-1$ where $p = p_C$ or p_T

After 96 hours, control and test tank mortalities are tabulated. Proportions are based on number of fish per replicate (e.g., 1/15 = 0.067). The critical F and t values are found in Tables 2 and 3, respectively.

Table 7. Summary for Modified T Test

	10 mg/L	100 mg/L
Calculated F statistic	The variances are considered statistically unequal because the control variance is zero. Therefore, the modified t test is used.	
Calculated t[1] statistic	-3.39	0.88
Critical t df[2] = (N-1)	3	3
Critical t *(see Table ~~2~~ 3)*	-1.64	-1.64
Does waste designate?	NO	YES
Waste code	not applicable	WT02

[1] 10 mg/L calculated t = (0.217-0-0.5)/[(0+0.0278)/4]$^{0.5}$ & 100 mg/L calculated t = (0.550 00.5)/[(0+0.0130)/4]$^{0.5}$

[2] (N - 1) is used in the modified t test when either $s_C^2 = 0$ or $s_T^2 = 0$

Zero variance in the control group dictates that the variances be considered unequal by the F test rule stated above. Therefore, the modified t test is employed and the calculated t for 10 mg/L is -3.39 and for 100 mg/L is 0.88. Because the 10 mg/L calculated t statistic (-3.39) is less than the critical t value (-1.64), H_o is rejected and the waste does **not** designate as extremely hazardous waste. However, because the 100 mg/L calculated t statistic (0.88) is greater than the critical t value (-1.64), H_o is **not** rejected and the waste **does** designate as dangerous waste.

Data Reporting

The report must contain the following information (a sample reporting form is found in Appendix E):

- Name of test, investigator, laboratory, and date test was begun.
- Detailed description of waste tested, its source, lot number, date of sample collection, known composition, known physical and chemical properties.
- Dilution water source, chemical characteristics, description of pretreatment, any additives.
- Detailed information about test organisms to include scientific name and how verified, wet weight, blotted dry, standard length for fish, age, life stage, source, history, observed diseases, treatments, acclimation procedure, food used.
- Description of experimental design and test chambers, test solution volume depth in chambers, number fish per replicate and treatment, loading, and lighting description.
- Description of any aeration performed before or during test.
- Definition of criterion used to determine endpoints/effects, summary of general observations.
- Number and percentage of fish deaths, per replicate and per treatment, in control and test chambers on a daily basis.
- Date run, identification and concentration of reference toxicant used and resulting mortality and LC_{50}.

- Methods used for and results with standard deviation of all chemical analyses of water quality and toxicant concentration, including validation studies and reagent blanks.
- Value of following: acclimation temperature, test temperature, pH, hardness, alkalinity, dissolved oxygen, and conductivity.
- Anything unusual about the test, any deviations from procedures, and any other relevant information.

Part B: Method 80-12
Acute Oral Rat Toxicity Test

Introduction

The acute oral rat bioassay is generally used to determine the relative acute toxicity of an unknown substance. This acute oral rat bioassay was developed by the Washington Department of Ecology (Ecology) for the purpose of determining if a waste meets the definition of dangerous waste in the *Dangerous Waste Regulations*, Chapter 173-303 WAC.

This method determines lethality and signs of acute toxicity from a waste sample administered in a single dose by gavage to a limited number of rats. The bioassay determines if the test sample exhibits a median lethal dose (LD_{50}) either greater than or less than or equal to a regulatory threshold corresponding to a waste designation, dangerous waste, special waste, or extremely hazardous waste. Applicable regulatory thresholds for dangerous waste, special waste, and extremely hazardous waste are doses of 5000, 500, and 50 mg/kg (weight of test substance per unit body weight), respectively. The waste sample fails the bioassay by exhibiting an LD_{50} statistically less than or equal to (i.e., toxicity greater than or equal to) the pertinent regulatory threshold.

Methods

Methodology has been adapted from acute oral toxicity testing, outlined by the EPA in the Code of Federal Regulations (CFR, 1993) and by Committee E-35 on Pesticides with the American Society for Testing and Materials (ASTM, 1994a; ASTM, 1994b).

Sample Collection and Preparation

A representative sample of waste should be obtained, using methods approved by Ecology. The waste sample should be homogenized and either dissolved or suspended in an appropriate vehicle, so that the material is in a homogeneous liquid form amenable to gavage. In decreasing order of preference, water, saline, or oil (e.g., corn oil) are recommended for the vehicle. The test solution concentration (i.e., weight of waste sample per volume solution) should not exceed 1 mL/100 g body weight, except when an aqueous solution is used, where 2 mL/100 g is acceptable (CFR, 1993).

The vehicle and test solution should be at the same temperature as the test room. Record the type of vehicle, test solution concentration, and temperature and photoperiod of the testing room.

Test Animals

Use common laboratory strains of albino rats (e.g., Sprague-Dawley) weighing 190 to 300 g, prefasted. At each dose level tested (5000, 500, or 50 mg/kg), use a minimum of five males and five females. Larger samples are allowed; maintain an equal number of male and female rats. Include the same number of animals, and equal numbers of males and females, in the vehicle control group as in the test group. Female rats should be nulliparous and nonpregnant. Use both males and females to incorporate possible gender differences in acute toxicity (Dixon, 1965) and renal metabolism (EPA, 1991b).

Pretest Conditioning

Animals should not have been used for any other tests and should be maintained according to accepted laboratory practices for the care and handling of test animals. Identify and cage individually each animal. Rat chow (or equivalent) and water must be available ad libitum until 18 to 20 hours before test initiation, at which time deprive animals of food. Examine each animal for overt signs of disease. Acclimate to the test environment for a minimum of seven days. During acclimation, observe animals for adverse health effects. Any animals demonstrating signs of spontaneous disease should be eliminated prior to test initiation. Use only animals judged to be healthy.

Procedure

Administer doses of 5000, 500, or 50 mg/kg to evaluate whether the waste sample designates as dangerous waste, special waste, or extremely hazardous waste, respectively. This dose is expressed as weight of the test substance per unit body weight. Determine the fasted body weight of each rat at the start of the test to calculate the volume of test solution (i.e., waste sample in vehicle) needed to deliver the desired dose.

Administer the test solution in a single dose by gavage, using a stomach tube or suitable intubation cannula. As stated above, the volume of test solution should not exceed 1 mL/100 g body weight, except when an aqueous solution is used, where 2 mL/100 g is acceptable (CFR, 1993). Deliver a uniform dose to rats with varying body weights by adjusting the volume of the prepared test solution of known waste concentration.

A concurrent vehicle control group should be treated identically to the test group with the notable exception that only vehicle (i.e., no waste sample) should be administered. Control group animals should receive a volume of vehicle per unit body weight (mL/kg) equal to the volume of test solution per unit body weight (mL/kg) administered to the test group animals. Record the volumes of vehicle and test solution administered to control and test group animals, respectively.

After dosing, immediately return all animals to individual cages with ad libitum food and water. Observe animals for mortality and signs of acute toxicity immediately after dosing, at one and four hours after dosing, and daily thereafter for 14 consecutive days. Cage-side observations should include evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior patterns. Particular attention should be directed toward noting tremors, convulsions, lethargy, salivation, and diarrhea. Record any mortalities and sign of acute toxicity.

Temperature of the testing room should be recorded daily. Body weights should be determined and recorded at the start of the test, on day 7, and at death.

Sacrifice surviving animals at the end of the test on day 14. Perform gross necropsies on all animals in both control and test groups. Record all gross pathology changes.

Calculation of Results

The bioassay result is calculated using the statistical method described below. Three examples are also presented. Within a 90 percent confidence limit, the test sample fails the bioassay by exhibiting a median lethal dose (LD_{50}) less than or equal to (i.e., toxicity greater than or equal to) the regulatory threshold (i.e., 5000, 500, or 50 mg/kg) when the cumulative binomial probability exceeds the established alpha level for small samples ($N < 30$), or when the calculated Z statistic is greater than -1.28 for larger samples ($N \geq 30$).

Approach

The frequency of samples displaying varying proportions of dead and live rats, as determined in acute lethality bioassays, is described by the binomial distribution. Due to the statistical independence among observations on individually caged rats, the experimental unit is the individual rat, and results from the dangerous waste oral rat bioassay can be analyzed with a binomial goodness of fit test. The observed proportion of mortalities in the test group is compared to a constant, $p_o = 0.5$, which corresponds to the LD_{50}. A one-tailed test is employed, since we wish only to determine if the sample LD_{50} is significantly greater than the regulatory threshold. This objective is consistent with the procedure for waste designation from bioassay data outlined in WAC 173-303-100(5)(c).

Two binomial methods are applied, depending on sample size (N) of the test group. Both methods incorporate a correction for up to 10 percent mortality in the control group. For $N < 30$, the cumulative binomial probability of mortalities is determined directly from the binomial distribution. For $N \geq 30$, the normal approximation to the binomial test is employed (Zar, 1984).

Hypothesis

In terms of concentration, the one-tailed null hypothesis (H_o) to be tested is $LD_{50} \leq 5000$ mg/kg (dangerous waste threshold), $LD_{50} \leq 500$ mg/kg (special waste threshold), or $LD_{50} \leq 50$ mg/kg (extremely hazardous waste threshold). In terms of mortality, each of these hypotheses corresponds to a one-tailed H_o: $p_T^a \geq p_o$, where p_T^a is the proportion of control-adjusted mortalities in the test group and $p_o = 0.5$ is the proportion of mortalities at the LD_{50}. An alpha level of 0.10 is used (i.e., one-tailed 90 percent confidence level), consistent with statistical methodology outlined by EPA (1986). Rejecting H_o demonstrates that the sample does not designate as a regulated waste.

Calculation (N < 30)

1) Minimum sample size

A minimum of 10 rats (N = 10) is to be used each in the control and test group with an equal number of males and females.

2) Control correction

The bioassay is invalid if control mortalities exceed 10 percent (EPA, 2002). Corrections for up to 10 percent mortality in the control are applied with Abbott's formula (Finney, 1971; EPA, 2002):

$$p_T^a = (p_T - p_C) / (1 - p_C)$$

where: p_T^a = control-adjusted proportion of mortalities in test group
p_T = observed proportion of mortalities in test group
p_C = observed proportion of mortalities in control group

Note that $p_T^a = p_T$ when $p_C = 0$. If $p_C \leq 0.10$ and $p_C > p_T$, then $p_T^a = 0$ as a result of data smoothing (EPA, 2002). That is, p_T and p_C are each set equal to their average, $(p_T + p_C) / 2$, so that $p_T^a = 0$.

3) Binomial probability

The cumulative binomial probability for the control-adjusted number of mortalities in the test group is determined by summing the discrete probabilities associated with all mortalities from zero to Np_T^a, where Np_T^a = control-adjusted number of mortalities. Np_T^a may be non-integer, in which case, the next

highest integer should be used. Discrete binomial probabilities can be obtained from statistical tables or from the following equation (Zar, 1984):

$$P(X) = [N! / X!(N - X)!]p_o^X(1 - p_o)^{(N - X)} \text{ (general equation)}$$

or

$$P(X) = [N! / X!(N - X)!]0.5^N$$

where: $P(X)$ = discrete probability of X mortalities in test group
N = number of rats in test group
X = number of mortalities in test group
$p_o = 0.5$ = proportion of mortalities at the LD_{50}

If the resultant cumulative binomial probability is less than or equal to alpha (≤ 0.10), then H_o is rejected and it is concluded that the waste does not designate as a regulated waste. Discrete and cumulative binomial probabilities for $p_o = 0.5$ and several sample sizes are presented in Table 11 below.

Table 11. Discrete and Cumulative Binomial Probabilities for $p_o = 0.5$

Discrete (D) and Cumulative (C) Binomial Probabilities for $p_o = 0.5$ and N = 10, 15, 20, and 25.

X	N = 10		N = 15		N = 20		N = 25	
	D	C	D	C	D	C	D	C
0	0.0010	0.0010	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0098	0.0108	0.0005	0.0005	0.0000	0.0000	0.0000	0.0000
2	0.0439	0.0547	0.0032	0.0037	0.0002	0.0002	0.0000	0.0000
3	0.1172	0.1719	0.0139	0.0176	0.0011	0.0013	0.0001	0.0001
4	0.2051	0.3770	0.0417	0.0593	0.0046	0.0059	0.0004	0.0005
5	0.2461	0.6231	0.0916	0.1509	0.0148	0.0207	0.0016	0.0021
6	0.2051	0.8282	0.1527	0.3036	0.0370	0.0577	0.0053	0.0074
7	0.1172	0.9454	0.1964	0.5000	0.0739	0.1316	0.0143	0.0217
8	0.0439	0.9893	0.1964	0.6964	0.1201	0.2517	0.0322	0.0539
9	0.0098	0.9991	0.1527	0.8491	0.1602	0.4119	0.0609	0.1148
10	0.0010	1.0000	0.0916	0.9407	0.1762	0.5881	0.0974	0.2122
11			0.0417	0.9824	0.1602	0.7483	0.1328	0.3450
12			0.0139	0.9963	0.1201	0.8684	0.1550	0.5000
13			0.0032	0.9995	0.0739	0.9423	0.1550	0.6550
14			0.0005	1.0000	0.0370	0.9793	0.1328	0.7878
15			0.0000	1.0000	0.0148	0.9941	0.0974	0.8852
16					0.0046	0.9987	0.0609	0.9461
17					0.0011	0.9998	0.0322	0.9783
18					0.0002	1.0000	0.0143	0.9926
19					0.0000	1.0000	0.0053	0.9979
20					0.0000	1.0000	0.0016	0.9995
21							0.0004	0.9999
22							0.0001	1.0000
23							0.0000	1.0000
24							0.0000	1.0000
25							0.0000	1.0000

Example 1

Ten rats each in the control and test group (N = 10) are orally dosed at 0 and 5000 mg/kg of waste, respectively, according to the bioassay procedure to determine if the waste sample designates as a

dangerous waste. After 14 days, 3 rats have died in the test group ($p_T = 0.300$) and zero control mortalities ($p_C = 0$) are observed. Therefore, $p_T = p_T^a$ and $Np_T^a = (10)(0.300) = 3$. For $p_o = 0.5$ and N = 10, discrete binomial probabilities for 0, 1, 2, and 3 deaths are 0.0010, 0.0098, 0.0439, and 0.1172, respectively (Table 1). Summing these probabilities, it is apparent that the cumulative probability for ≤ 3 deaths is greater than the established alpha level of 0.10 (i.e., 0.1719 > 0.10). Therefore, H_o is not rejected and the waste designates as a dangerous waste.

Example 2

Twenty rats each in the control and test group (N = 20) are orally dosed at 0 and 5000 mg/kg of waste, respectively, according to the bioassay procedure in order to determine if the waste sample designates as a dangerous waste. After 14 days, 7 rats have died in the test group ($p_T = 0.350$) and 2 control mortalities ($p_C = 0.100$) are observed. Therefore, $p_T^a = 0.278$ and $Np_T^a = (20)(0.278) = 5.56$. Np_T^a is then rounded to 6 deaths, as described above. For $p_o = 0.5$ and N = 20, discrete binomial probabilities for 0, 1, 2, 3, 4, 5, and 6 deaths are 0, 0, 0.0002, 0.0011, 0.0046, 0.0148, and 0.0370, respectively (Table 1). Summing these probabilities, it is apparent that the cumulative probability for ≤ 6 deaths is less than the established alpha level of 0.10 (i.e., 0.0577 < 0.10). Therefore, H_o is rejected and the waste does not designate as a dangerous waste.

Calculation (N = 30)

1) Control correction

The bioassay is invalid if control mortalities exceed 10 percent (EPA, 993). The control correction is performed with Abbott's formula as described above.

2) Normal approximation

Based on the mean and standard deviation for a binomial distribution, the following formula has been adapted from Zar (1984) to evaluate how p_T^a differs from $p_o = 0.5$ in terms of a normal distribution:

$$Z = (p_T^a - p_o) / [p_T^a(1 - p_T^a)/N]^{0.5} \quad \text{(general equation)}$$

or

$$Z = (p_T^a - 0.5) / [p_T^a(1 - p_T^a)/N]^{0.5}$$

where: Z = normal deviate

$p_o = 0.5$ = proportion of mortalities at the LD_{50}

N = number of rats in test group

The calculated Z test statistic is compared to a one-tailed critical Z value at an alpha level of 0.10 (i.e., Z = -1.28). When the calculated Z value is ≤ -1.28, H_o is rejected, and it is concluded that the waste does not designate as a regulated waste.

Example 3

Fifty rats per control and test groups (N = 50) are orally dosed at 0 and 5000 mg/kg of waste, respectively, according to the bioassay procedure in order to determine if the waste sample designates as a dangerous waste. After 14 days, 20 rats have died in the test group ($p_T = 0.400$) and 4 control mortalities ($p_C = 0.080$) are observed. From these data, $p_T^a = 0.348$ and Z = -2.26. Because the calculated Z statistic (-2.26) is less than the critical Z value (-1.28), H_o is rejected and the waste does not designate as dangerous waste.

Data Reporting

The report must contain the following information:

- Study initiation and termination dates.
- Description of testing facility.
- Source, genetic strain, and sex of control and test animals.
- Description of sample preparation, including type of vehicle used and test solution concentration.
- Dose of test solution and volumes of vehicle and test solution administered to respective control and test groups.
- Animal body weights at test initiation, day 7, and at death in both control and test group.
- Mortalities and signs of acute toxicity immediately after dosing, at one and four hours after dosing, and daily thereafter for 14 consecutive days in both control and test groups.
- Photoperiod and daily temperature of testing room.
- Gross necropsy results.
- Statistical analysis to evaluate whether the waste sample LD_{50} is either greater than or less than or equal to the administered dose which corresponds to an established regulatory threshold.
- Statement of whether the waste sample passes or fails the bioassay and designates as a dangerous waste, special waste, or extremely hazardous waste, according to the *Dangerous Waste Regulations*, Chapter 173-303 WAC.

References

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Appendix A

Neutralized Fish Toxicity Test Procedure

The neutralized fish toxicity test procedure is identical to the Part A method except as noted below. The only difference is that aquarium water is neutralized after the waste is introduced into the aquarium, and before the fish are placed into the aquarium.

The aquarium water may be neutralized using only aqueous hydrochloric acid (HCl) or sodium hydroxide (NaOH). Add the neutralizing solution dropwise while stirring the contents of the test tank, and measuring the pH continuously. The pH of the aquarium water should be adjusted to 7.5 and allowed to stand 30 minutes to detect any pH drift. If the pH drifts outside the range 7.2 - 7.8, the aquarium water should be readjusted to pH 7.5, and the fish introduced immediately thereafter. Further pH adjustment is not allowed.

The neutralizing solutions should be as concentrated as practicable to minimize diluting the aquarium water. Under no circumstances should > 50 mL be used to adjust pH. Report the actual volume and normality of neutralizing solution used on the data reporting sheet.

Conducting the Neutralized Fish Toxicity Test

The neutralized fish toxicity test requires all of the proceeding steps for validity. Conduct steps 1 - 5 in the sequence shown.

1. Fill the test tanks and control tanks with dilution water allowing for 400 mL of water from the sample extraction step. The dilution water must show hardness, alkalinity, and pH within the range for "soft" to "moderately hard" water as described in Table 1.
2. Introduce the waste into the test tanks. The waste includes any undissolved sample, the extract, the rinse, and the extraction flask or bottle.
3. Measure and record the pH.
4. Adjust the pH to 7.5 and wait 30 minutes. If the pH drifts outside the range 7.2-7.8, re-adjust the pH and proceed immediately to steps 5, 6, and 7.
5. Collect a sample for measuring hardness, alkalinity, and conductivity.
6. Measure and record dissolved oxygen, pH, and temperature.
7. Introduce the fish into the test tanks and control tanks.
8. Observe and record data as indicated on the data sheet.
9. Maintain temperature and dissolved oxygen as described in the section on "Test Procedure."
10. Terminate the test after 96 hours.
11. Complete the data reporting sheet.

Recording the Data

For neutralized tests, record the following information on the data reporting sheet:

- Neutralizer (i.e., NaOH or HCl).
- Normality of neutralizer.
- Volume of neutralizer.
- Indicate if pH readjustment was necessary 30 minutes after initial pH adjustment.

Appendix B

Good Laboratory Standards

The following was adapted from EPA's Good Laboratory Practice (GLP) Standards. These standards are recommended, not required. Laboratories are encouraged to develop QA/QC programs, which address these recommended standards.

Organization and Personnel: Personnel

Each individual engaged in the conduct of or responsible for the supervision of a study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned functions.

Each testing facility shall maintain a current summary of training and experience and job description for each individual engaged in or supervising the conduct of a study.

There shall be a sufficient number of personnel for the timely and proper conduct of the study according to the protocol.

Personnel shall take necessary personal sanitation and health precautions designed to avoid contamination of test and control substances and test systems.

Personnel engaged in a study shall wear clothing appropriate for the duties they perform. Such clothing shall be changed as often as necessary to prevent microbiological, radiological, or chemical contamination of test systems and test and control substances.

Any individual found at any time to have an illness that may adversely affect the quality and integrity of the study shall be excluded from direct contact with test systems, test and control substances and any other operation or function that may adversely affect the study until the condition is corrected. All personnel shall be instructed to report to their immediate supervisors any health or medical conditions that may reasonably be considered to have an adverse effect on a study.

Biologist in Charge

For each study, a biologist, or a scientist, or other professional of appropriate education, training, and experience, or combination thereof, shall be identified as the biologist in charge. The biologist in charge has overall responsibility for the technical conduct of the study, as well as for the interpretation, analysis, documentation, and reporting of results, and represents the single point of study control. The biologist in charge shall assure that:

- The protocol, including any change, is approved as provided by Ecology and is followed.
- All experimental data, including observations of unanticipated responses of the test system are accurately recorded and verified.

Unforeseen circumstances that may affect the quality and integrity of the study are noted when they occur, and corrective action is taken and documented.

- Test systems are as specified in the protocol.
- All applicable good laboratory practice regulations are followed.
- All raw data, documentation, protocols, specimens, and final reports are transferred to the archives during or at the close of the study.

Protocol

Each study shall have an approved written protocol that clearly indicates the objectives and all methods for the conduct of the study. The protocol shall contain but not ~~be~~ necessarily be limited to the following information:

- A descriptive title and statement of the purpose of the study.
- Identification of the test and control substance by chemical name, Chemical Abstract Service Registry Number (CAS), or other established identification system.
- The name and address of the sponsor and the name and address of the testing facility at which the study is being conducted.
- The proposed starting and completion dates.
- Justification for selection of the test system.
- Where applicable, the number, body weight range, sex, source of supply, species, strain, substrain, and age of the test system.
- The procedure for identification of the test system.
- A description of the experimental design, including the methods for the control of bias.
- A description ~~and/~~or identification of the diet used in the study as well as solvents, emulsifiers ~~and/~~or other materials used to solubilize or suspend the test or control substances before mixing with the carrier. The description shall include specifications for acceptable levels of contaminants that are reasonably expected to be present in the dietary materials and are known to be capable of interfering with the purpose or conduct of the study if present at levels greater than established by the specifications.
- The route of administration and the reason for its choice.
- Each dosage level, expressed in milligrams per kilogram of body weight or other appropriate units, of the test or control substance to be administered and the method and frequency of administration.
- Method by which the degree of absorption of the test and control substances by the test system will be determined if necessary to achieve the objectives of the study.
- The type and frequency of tests, analyses, and measurements to be made.
- The records to be maintained.

- The date of approval of the protocol by the sponsor and the signature of the biologist in charge.
- A statement of the proposed statistical methods to be used.
- All changes in or revisions of an approved protocol and the reasons therefore shall be documented, signed by the biologist in charge, dated, and maintained with the protocol.

Conduct of a Study

The study shall be conducted in accordance with the protocol.

The test systems shall be monitored in conformity with the protocol. Specimens shall be identified by test system, study, nature, and date of collection. This information shall be located on the specimen container or shall accompany the specimen in a manner that precludes error in the recording and storage of data.

Records of gross findings for a specimen from postmortem observations shall be available to a pathologist when examining that specimen histopathologically.

All data generated during the conduct of a study, except those that are generated as direct computer input, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed or identified at the time of the change. In computer driven data collection systems, the individual responsible for direct data input shall be identified at the time of data input. Any change in computer entries shall be made so as not to obscure the original entry, shall indicate the reason for change, and shall be dated and the responsible individual shall be identified.

Maintenance and Calibration of Equipment

Equipment shall be adequately inspected, cleaned, and maintained. Equipment used for the generation, measurement, or assessment of data shall be adequately tested, calibrated, or standardized.

Written standard operating procedures (SOPs) shall set forth in sufficient detail the methods, materials, and schedules to be used in the routine inspection, cleaning, maintenance, testing, calibration, or standardization of equipment, and shall specify remedial action to be taken in the event of failure or malfunction of equipment. The written SOPs shall designate the person responsible for the performance of each operation, and copies of the standard operating procedures shall be made available to laboratory personnel.

Written records shall be maintained of all inspection, maintenance, testing, calibrating, or standardizing operations. These records, containing the date of the operation, shall describe whether the maintenance operations were routine and followed the written standard operating procedures. Written records shall be kept of non-routine repairs performed on equipment as a result of failure and malfunction. Such records shall document the nature of the defect, how and when the defect was discovered, and any remedial action taken in response to the defect.

Testing Facilities Operation: Standard Operating Procedures

A testing facility shall have standard operating procedures in writing setting forth study methods that management is satisfied are adequate to ensure the quality and integrity of the data generated in the course of a study. All deviations in a study from standard operating procedures shall be authorized by the study director and shall be documented in the raw data. Significant changes in established standard operating procedures shall be properly authorized in writing by management.

SOPs shall be established for, but not limited to, the following:

- Animal room preparation.
- Animal care.
- Receipt, identification, storage, handling, mixing, and method of sampling of the test and control substances.
- Test system observations.
- Laboratory tests.
- Handling of animals found moribund or dead during study.
- Necrospy of animals or postmortem examination of animals.
- Collection and identification of specimens.
- Histopathology.
- Data handling, storage, and retrieval.
- Maintenance and calibration of equipment.
- Transfer, proper placement, and identification of animals.
- Each laboratory area shall have immediately available laboratory manuals and SOPs pertinent to the laboratory procedures being performed (e.g., toxicology, histology, clinical chemistry, hematology, teratology, necropsy). Published literature may be used as a supplement to standard operating procedures.
- Historical file of SOPs, and all revisions thereof, including the dates of such revisions, shall be maintained.

Reagents and Solutions

All reagents in the laboratory areas shall be labeled to indicate identity, titer or concentration, storage requirements, and expiration date. Solutions prepared in the laboratory shall be labeled to indicate identify, concentration, date of preparation, and preparer's name or initials. Deteriorated or outdated reagents and solutions shall not be used.

Appendix C

Water Quality

Water quality parameters, in addition to pH, hardness, and alkalinity, can adversely affect the outcome of acute fish toxicity tests. Sub-lethal concentrations of certain materials in dilution water may contribute to fish mortality because of the additive effect of similar materials in the waste being tested, or because of synergism resulting from dissimilar materials. The more common materials and parameters of concern are presented below with recommended ranges or maximum concentrations. If erratic results are experienced in the toxicity test, the dilution water should be checked for the materials included in Table 8, or for other materials, which may be present in the dilution water source. Also refer to EPA-821-R-02-012 Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms for additional guidance on water quality.

Table 8. Water Quality Parameters[a]

PARAMETER	RECOMMENDED
pH	7.2 - 7.8
Alkalinity	30 - 70 mg/L as $CaCO_3$
Ammonia (NH_3)	0.02 mg/L unionized
Cadmium (Cd)	100 ng/L
Chloride (Cl)	2 - 3 mg/L
Copper (Cu)	less than 5 µg/L
Hardness	40-100 mg/L as $CaCO_3$
Lead (Pb)	0.03 mg/L or less
Mercury (Hg) organic or inorganic	100 ng/L
Nickel (Ni)	88 µg/L or less
Nitrogen (N)	maximum total gas pressure 110 percent of saturation
Nitrite (NO_2)	100 µg/L
Oxygen (O_2)	at least 8 mg/L at 12^{o}C
Ozone (O_3) Residual	3 µg/L
PCB as Aroclor 1254	50 ng/L or less
Total Suspended and Settleable Solids	80 mg/L or less
Zinc (Zn)	less than 65 µg/L
Total organochlorine pesticides	50 ng/L or less

[a]Adapted from Wedemeyer et al. (1981) and EPA (2002).

Appendix D

Table 9. Approximate Saturation[1] and Concentration Values of Dissolved Oxygen in Freshwater at 12°C

Altitude (ft)	DO (mg/L) at 100% Saturation	DO (mg/L) at 80% Saturation	DO (mg/L) at 60% Saturation	Altitude (ft)	DO (mg/L) at 100% Saturation	DO (mg/L) at 80% Saturation	DO (mg/L) at 60% Saturation
0	10.71	8.57	6.43	4000	9.35	7.48	5.61
100	10.68	8.54	6.41	4250	9.26	7.41	5.56
250	10.62	8.50	6.37	4500	9.18	7.34	5.51
500	10.54	8.43	6.32	4750	9.09	7.27	5.45
750	10.45	8.36	6.27	5000	9.01	7.21	5.41
1000	10.37	8.30	6.22	5250	8.92	7.14	5.35
1250	10.28	8.22	6.17	5500	8.84	7.07	5.30
1500	10.20	8.16	6.12	5750	8.75	7.00	5.25
1750	10.11	8.09	6.07	6000	8.67	6.94	5.20
2000	10.03	8.02	6.02	6250	8.58	6.86	5.15
2250	9.94	7.95	5.96	6500	8.50	6.80	5.10
2500	9.86	7.89	5.92	6750	8.41	6.73	5.05
2750	9.77	7.82	5.86	7000	8.33	6.66	5.00
3000	9.69	7.75	5.81	7250	8.24	6.59	4.94
3250	9.60	7.68	5.76	7500	8.16	6.53	4.90
3500	9.52	7.62	5.71	7750	8.07	6.46	4.84
3750	9.43	7.54	5.66	8000	7.99	6.39	4.79

[1] Based on Pillard (1996).

Appendix E

Recommended Fish Loading

Prior to use in toxicity tests, fish are usually maintained at the test facility in a circulated holding tank. A minimum level of circulation is required to maintain the viability of test fish. The recommended level of circulation varies depending on the species of fish, size of fish, and temperature. For small fish, the size of the fish is usually reported in terms of number of fish per pound. The table below lists the recommended fish minute loadings (pounds of fish per gallon per minute of inflow), to promote the health and quality of salmonids used in toxicity tests.

Table 10. Recommended Fish Loadings[a]

SPECIES	WATER TEMPERATURE		POUNDS FISH PER GPM		
	°C	°F	1000[b]	500[b]	100[b]
Coho Salmon	3	38	3.5	5.0	8.0
	9	48	2.7	4.0	6.0
	14	58	2.2	3.0	4.5
	17	63	----	2.0	3.5
Fall/Spring Chinook Salmon	3	38	3.0	4.0	6.0
	9	48	2.5	3.0	5.0
	14	58	2.0	2.2	3.5
	17	63	----	1.2	3.0

[a] Adapted from Wedemeyer & Wood (1977)
[b] Fish size (number of fish per pound)

Appendix F

Sample Data Sheet

The *Data Sheet for Static Basic Acute Fish Toxicity Test* is printed on page 37. Please copy this form to report test data.

DATA SHEET FOR STATIC BASIC ACUTE FISH TOXICITY TEST

Laboratory _____
Analyst _____

Industry/Toxicant _____ Beginning Date _____ Time _____
Address _____ Ending Date _____ Time _____
Collector _____ Test Organism _____
Date Sample Collected _____ Required Test Temperature Range _____

Laboratory Reference Number	Test Container No.	Conc. (mg/l)	Number of Cumulative Deaths					Dissolved Oxygen (mg/l)					pH 25 C					Temperature (C)					Total Hardness (mg/l as $CaCO_3$)		Total Alkalinity (mg/l as $CaCO_3$)		Conductivity µMHOS/cm	
			0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	96	0	96	0	96

Sample Description _____
Average Weight _____ Mean Length _____ Longest _____ Shortest _____ Ratio (long/short) _____
Number of organisms per chamber _____ Ratio of flesh to water _____
Comments_____
_____

Method on File with the Department of Ecology

GENERAL PROCEDURE FOR STATIC BASIC ACUTE FISH TOXICITY TEST Data Verified By _____

Date _____

Appendix G

Recommended Test Conditions and Procedures for Fish Bioassay

Test type	• Static non-renewal
Duration	• 96 hours
Light quality	• Ambient laboratory illumination
Light intensity	• 10-20 uE/m^2/s (50-100 ft-c)
Photoperiod	• 16 hours light, 8 hours darkness
Test chamber size	• 3.8 L or 1 gal minimum
Minimum depth	• 15 cm test solution
Test solution renewal	• none
Source of fish	• hatchery stock, free of known diseases
Control water	• moderately hard water; see Table 1
Fish age	• 30 - 90 days
Temperature	• 12 ^{o}C $\pm$ 1^{o}C
Oxygen/aeration	• None unless DO<80 percent saturation at altitude; ideal DO is 80 to 100 percent saturation
pH	• no pH adjustment
Feeding	• not required
Cleaning	• not required
Number of fish per test chamber	• minimum 10
Number of replicate chamber per concentration	• minimum 3
Number of fish per concentration	• minimum 30
Observations	• fish mortality, appearance, and behavior at 24, 48, 72, and 96 hours minimum
Measurements	• temperature, DO, pH, in each tank daily; conductivity test
Endpoints	• mortality
Test acceptability	• 90 percent survival or greater in controls
Reference toxicant	• $CuSo_4$, $CdC1_2$, KCl, NaCl, or $ZnSO_4$ (not inclusive); evaluate at least once/month and with each new batch of fish
Solvents	• Extraction water is the control water used in test controls

Concentration	• 100 mg/L to determine dangerous waste; 10 mg/L to determine extremely hazardous waste
Control water	• Moderately hard water
Sampling date	• Date of sample collection; tests done on sample collected more than 45 days prior to extraction must be noted in the report
Transport and storage	• Extract within seven days of sample receipt; store in dark at 4°C; test within 8 days of sample collection
Extraction	• Extract using rotary agitation device (30 $\pm$ 2 rpm) for 18 hours $\pm$ 2 hours at 23°C $\pm$ 2°C

Glossary

^{o}C	degree(s) Celsius
$CaCO_3$	calcium carbonate
$CaSO_4$	calcium sulfate
$CaSO_4 \bullet 2H_2O$	calcium sulfate dihydrate (gypsum)
cm	centimeter
CO_2	carbon dioxide
COD	chemical oxygen demand
d	day
DO	dissolved oxygen
DW	dangerous waste
EHW	extremely hazardous waste
^{o}F	degree(s) Fahrenheit
g	grams
gal	gallons
gpm	gallons per minute
h	hour
HCL	hydrochloric acid
H_2O	water
in	inch(es)
KCL	potassium chloride
L	liter
m	meter
mg	milligram
mg/L	milligram per liter
$MgSO_4$	magnesium sulfate
min	minute(s)
mL	milliliter
mg/kg	milligram per kilogram
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
ng/L	nanograms per liter
NH_3	ammonia
QA/QC	quality assurance/quality control
qt	quart
rpm	rotations per minute
TOC	total organic carbon

WAC	Washington Administrative Code
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to

Terminology

Grammatical Terms

Can…means "is/are able to"

May…means "is/are allowed to"

Might…means "could possible;" never used as a synonym for "may" or "can"

Must…expresses an absolute requirement

Should…means the specified condition is recommended and ought to be met if possible

Technical Terms

Acclimatize…to become physiologically adapted to a particular level of one or more environmental variables, such as temperature or water chemistry.

Acute toxicity…a discernible, adverse effect (lethal or sublethal) induced in the test population within a short period of exposure to a test material, usually constituting a non-substantial portion of their life span.

Conductivity…is a numerical expression of an aqueous solution's ability to carry an electrical current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution temperature. Conductivity is normally reported in the Système International d'unités (SI) unit of millisiemens/meter, or as micromhos/centimeter (1 mS/m = 10 µmhos/cm).

Control…a treatment that is essentially free of contaminants and is used routinely to assess the acceptability of a test. In Salmonid tests, the control must duplicate all conditions of the exposure treatment(s), but must contain no test material.

Dangerous waste …those solid wastes designated per WAC 173-303-070 through 173-303-100 as dangerous, extremely hazardous, or mixed waste.

Deionized water…water passed through resin columns to remove ions.

Distilled water…water passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Extremely hazardous waste…those dangerous and mixed wastes designated per WAC 173-303-100 as extremely hazardous. Also, those dangerous wastes showing statistically significant mortality in test concentrations of 10 mg/L.

Flow-through…continuous renewal of holding water or test solution.

Hardness…a measure of the concentration of calcium and magnesium ions in water, expressed as mg/L calcium carbonate ($CaCO_3$) or equivalent.

LC_{50}…the median lethal concentration (i.e., the concentration of material in water estimated to be lethal to 50 percent of the test organisms). The LC_{50} and its 95 percent confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96 hour LC_{50}).

Leachate…any liquid, including any components suspended in the liquid, that which has percolated through or drained from dangerous waste.

Lethal… means causing death by direct action. Death of fish is defined as the cessation of all visible signs of movement or other activity, such as no response from gentle prodding.

Loading…the weight of organisms per liter of test solution; this is limited to minimize the decrease in dissolved oxygen (DO) or toxicant below acceptable levels, the accumulation of injurious concentrations of metabolic waste products, or stress induced by crowding.

Lux…a unit of illumination equal to 1 lumen per square meter. One lux = equals 0.0929 foot-candles and one foot-candle = equals 10.76 lux.

Observations…routine checks of biological and water quality variables, which may include fish survival and behavior, water temperature, dissolved oxygen, pH, hardness and conductivity.

pH…the negative logarithm of the hydrogen ions activity in gram equivalents per liter. The pH value expresses the degree of intensity of both acidic and alkaline solutions on a scale from 0 to 14, with 7 representing neutrality. Numbers less than 7 signify increasingly greater acidic solutions, and numbers greater than 7 indicate increasingly greater basic, or alkaline, solutions.

Photoperiod…duration of illumination and of darkness over a 24 hour day.

Reconstituted water...standard, synthetic water prepared with de-ionized water and reagent grade chemicals or mineral water; to be used for culturing of organisms, as control water, and as dilution water for test solutions.

Reference toxicant…a known toxicant used to measure all aspects of toxicity testing, including organism quality, presence and effects of stressors other than test compounds, and the quality of analytical techniques. Data from regular reference toxicant testing is used to establish the precision of bioassay results generated by the laboratory.

Replicate…Each of several experimental units that are tested simultaneously using the sample experimental conditions.

Static…describes toxicity tests in which test solutions are not renewed during testing.

Swim-up...stage at which Salmonids emerge from the gravel and begin to actively feed. It is the point at which to begin marking the age of the fish.

Toxicity...inherent potential or capacity of a material to cause adverse effects in living organisms.