Biological Testing Methods 80-12 For the Designation of Dangerous Waste

Static Acute Fish Toxicity Test

Hazardous Waste and Toxics Reduction Program

Washington State Department of Ecology
Olympia, Washington

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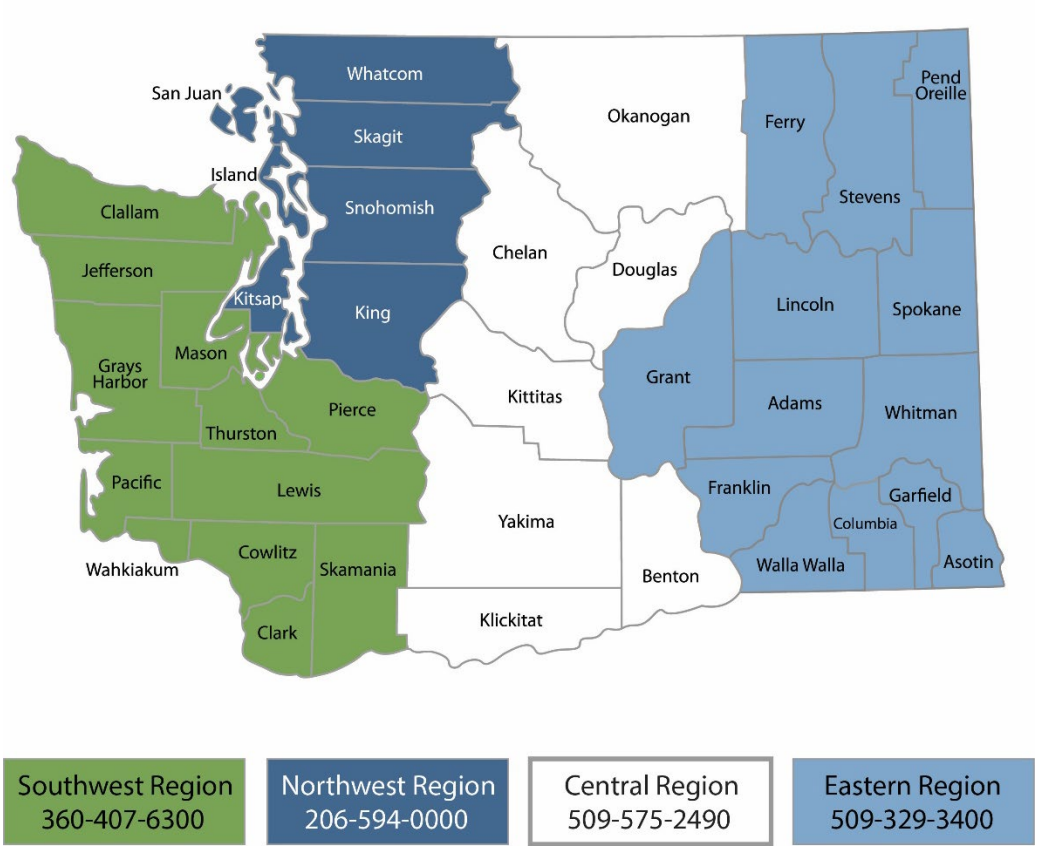

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Part A: Method 80-12
Static Acute Fish Toxicity Test

Introduction

The Washington State Department of Ecology (we, 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 in the Washington Administrative Code (WAC).[3]

Method 80-12 provides a simple, low-cost method to test waste toxicity 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 for dangerous waste (DW), 10 mg/L for extremely hazardous waste (EHW), or 10 mg/L for special waste. For this method's purposes, LC_{50} is the median lethal concentration of waste that kills 50% 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.[4]

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. 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.[5]

Health and safety

Developing and maintaining an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management. It includes:

- An appointed laboratory health and safety officer, who has the responsibility and authority to develop and maintain a safety program.
- A formal, written health and safety plan, which is provided to each laboratory staff member.
- An ongoing training program on laboratory safety.

[3] https://app.leg.wa.gov/WAC/default.aspx?cite=173-303
[4] https://app.leg.wa.gov/WAC/default.aspx?cite=173-303-110
[5] https://app.leg.wa.gov/WAC/default.aspx?cite=173-303-910

- Regularly scheduled and 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. We encourage testing laboratories to implement these and other recommendations to promote the health and safety of laboratory personnel.

- Test industrial wastes in compliance with accepted rules pertaining to the handling of hazardous materials. We recommend that personnel conducting toxicity tests not work alone.
- Consider wastes potential health hazards and minimize exposure to them.
- 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.
- Maintain good housekeeping habits to 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.
- Train staff in basic first aid and cardio-pulmonary resuscitation (CPR).
- Require all personnel to receive tetanus immunizations. The following are optional and dependent on the types of waste anticipated:
    - If suspected contact with human bodily fluids, including blood:
        - Hepatitis B
    - If suspected contact with human feces or urine:
        - Hepatitis A
        - Typhoid fever
        - Polio

          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.
- Properly handle and safely dispose wastes generated during toxicity testing. 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) in the References section.

Methods and Materials

Holding facilities

Facilities should include tanks for holding and acclimating test fish in a constant-temperature room or recirculating water bath for the test chambers. Water tanks and headboxes for holding, acclimation, and dilution should be equipped for temperature control and aeration. Air used for aeration must be free of oil and fumes; you may use filters to remove oil and water. The test facility should be well ventilated and free of fumes. Shield test fish from disturbances during holding, acclimation, and testing.

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% and 100% saturation; you may use gentle aeration if necessary to drive off the ammonia from generated biological waste.

A photoperiod of 16 hours of light and 8 hours of darkness should be provided during the test. Preferably, you should raise light intensity 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. Choose tanks and other equipment that contact stock solutions or test solutions 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 you will need strainers 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 centimeters (cm). Cover chambers to prevent test fish from jumping out. Test chambers should be chemically inert and selected to minimize sorption of test material. Commonly used vessel sizes in static tests using juvenile fish

are 3.8 liters (L) (i.e., 1 gallon [gal]) or 19.0 L (i.e., 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). If stainless steel is used to construct test chambers, weld the seams. **Do not solder them**.

You may use commercially available test chambers if they meet the above requirements. You may also use disposable, non-toxic plastic (e.g., polypropylene) liners. We **highly recommend** 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% concentrated nitric acid.
- Copious clean water.
- Reagent grade acetone.
- Copious clean water.

At every test termination, clean the chambers in this sequence:

- Empty the chambers.
- Immediately rinse with clean water.
- Clean 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).
- Rinse with copious clean water.

Acid is useful for removing mineral deposits and 200 milligram (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 milliliters (mL) of liquid household chlorine bleach to 1 L of water. **Do not use acid and hypochlorite together. The combination may produce hazardous chlorine fumes.**

You may also sterilize tanks 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.

To disinfect 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 hypochlorites are acutely toxic to most aquatic organisms. Remove hypochlorite by flushing with water or 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

You must have an adequate supply of quality dilution water 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% 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. You may use dechlorination using activated carbon filters or other methods to dechlorinate dilution water as thoroughly as possible. You may also remove chlorine using an activated carbon filter and 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.

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

Keep the dilution water dissolved oxygen between 90% and 100% saturation prior to solution preparation. If needed, aerate dilution water before solution preparation. Rinse test chambers with dilution water just before use.

Dilution water hardness can affect the outcome of the test. Use soft or moderately hard dilution water in this method. In no case should the dilution water's hardness be less than 40 mg/L CaCO3. Low hardness or 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*

Water Type	$NaHCO_3$ mg/L[a]	$CaSO_4 \bullet 2H_2O$ mg/L[a]	$MgSO_4$ mg/L[a]	KCl mg/L[a]	pH[b]	Total Resulting Hardness[c]	Total Resulting Alkalinity[c]
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

Notes:

- *Adapted from EPA (2002).
- a: Add reagent grade chemicals to distilled or deionized water.
- b: Approximate equilibrium pH after aerating 24 hours.
- c: 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)*
- Brook trout *(Salvelinus fontinalis)*

The test temperature for all species is **12 ± 2 °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 fresh-water fish are private, state, and federal hatcheries.

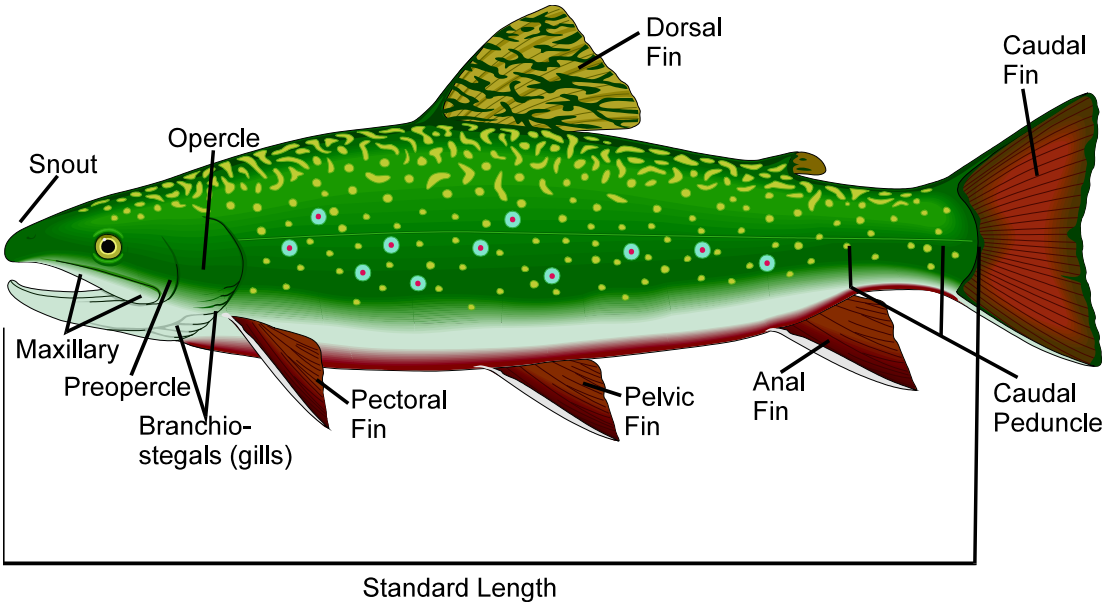

Figure 1: Fish diagram

Test fish should be juveniles (<3.0 grams [g]/fish) who are active feeders. Because fish will not be fed for 48 hours before testing, as well as during the test, juveniles should be of sufficient size to minimize nutritional stress during this period. Fish should be 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 difference. In any single test, all fish should be from the same batch.

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.)*
- 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 the Cleaning and Disinfection section.

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 degree Celsius (°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 are 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. Sterilize equipment used to handle aquatic organisms between uses by autoclaving or treating with an iodophor, 200 mg hypochlorite/L, or 30% formalin plus 1% benzalkonium chloride for at least 1 hour. Sterilize or wash hands before **and** after handling fish.

Holding and acclimation

After collection or transportation, quarantine and acclimate fish to laboratory conditions before use in tests. The recommended holding period is 14 days. However, if fish are obtained from a commercial hatchery and are certified disease free (per the performance standards specified in the chart below), an acclimation period of 48 hours is acceptable. Regardless of duration of the acclimation period, the batch of test organisms must achieve these performance standards.

Table 2: Performance standards for certified disease-free fish.

Mortality Observation	Action
> 10% mortality of batch	Reject entire batch
5–10% mortality of batch	Extend acclimation period for recommended 7 days
< 5% mortality of batch	Batch acceptable for testing

To maintain healthy organisms and avoid unnecessary stress during the acclimation period, fish must not be crowded or subjected to rapid changes in environmental conditions. Water used for acclimatization should be from the same source as used in the test. Any acclimatization to

dilution water should be done gradually over 48 hrs. Changes in water temperature should not exceed 3°C within any 24-hour period during holding or acclimatization (EPA, 2016).

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 it reduces the metabolic rate and the number and severity of disease outbreaks. Recommended fish loading for holding tanks is provided for various water temperatures, fish size, and species in Appendix C and Appendix D.

If stock fish have 5% 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

Carefully observe test fish 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, we recommend you remove the stressor and reacclimatize the test organisms for 7 days. The following physical characteristics indicate stress:

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

Sample preparation

Sample reporting requirements

All samples must be extracted using the rotary agitation 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 ± 2 hours at 23 ± 2°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 millimeter [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 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

Follow these steps for this method:

1. Weigh out the correct amount of a well-mixed sample and transfer into a 500 or 1000 mL extractor bottle.
2. Add 200 mL of test dilution water to the extractor bottle.
3. Place a Teflon cap liner over the mouth of the extractor bottle and screw on the cap.
4. Mix the sample on the rotary agitation apparatus for 18 ± 2 hours at 23 ± 2°C, remove the cap, and pour the extraction water into the dilution water in the fish test tank.
5. 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.
6. Begin the test as described in the Test Procedure section.
7. Exercise extreme care throughout the course of the extraction so as not to contaminate the exterior of the extraction flask. Acceptable methods include sealing sterile flasks in sterile aluminum foil prior to use or copiously rinsing with deionized water, at a minimum.

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% 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. Do not add any of the toxicants being tested to the control.

Test temperature

The test temperature for all test species is 12°C. The actual test temperature must not deviate from 12°C by more than ± 2.0°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.
- 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.

The pH may tend to drift during the 96-hour test period. This drift is due, in part, to fish respiration, which increases the CO_2 content of the water and 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 dissolved oxygen should not fall below 60% saturation (e.g., 6.4 mg/L at 12°C at 250 feet above sea level) during the test. If the dissolved oxygen falls below 50% saturation during the test and mortality exceeds the pass/fail threshold, you must void the test. Dilution water should be at or near 90–100% saturation. If dissolved oxygen levels are in danger of dropping below 60% saturation, gentle aeration is permitted (EPA, 2016). Please refer to Appendix B 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, re-suspends fecal matter, and increases volatilization. Because evaporation readily occurs at the surface, you can achieve efficient aeration 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 grams (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. We recommend lower loadings if dissolved oxygen does not remain above 60% saturation for the first 48 hours of the test and above 50% saturation after 48 hours.

Limit loading to ensure:

- Concentrations of dissolved oxygen and toxicant are not decreased below acceptable levels.
- Concentrations of metabolic products are not increased 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 test initiation. 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. Fish should be of sufficient size to survive and not exhibit signs of stress during this time period without food.

Duration and acceptability

Begin the test by placing the extraction vessel of waste and leachate in the test chamber on its side. Also place the cap in the test chamber.

The extraction vessel exterior should be sterile or as clean as possible to avoid introducing additional contamination. **Blank** extraction vessels 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% survival or greater for test acceptance or validity.

Test observations and measurements

Measure dissolved oxygen, temperature, pH, and conductivity in all test tanks at test initiation. Every 24 hours following test initiation, simultaneously measure dissolved oxygen, temperature, and pH in at least one randomly selected test tank in the control and at each concentration (as long as fish are present). Also record the dissolved oxygen, temperature, and pH at the end of the test or at the time the last fish dies in the test tank. You must measure hardness and alkalinity in the control and each test concentration at the start of the test. Obtain hardness and alkalinity measurements from test solutions prior to pouring into individual replicates, or from one replicate of each control and test concentration at test initiation.

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. We suggest 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.

Criterion for death

Death is the adverse effect most often used in acute toxicity tests. The criterion for death in fish is lack of movement, especially the absence of respiratory movements 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

Destroy all fish, both control and treatment, at termination of test, after data collection is complete.

Data analysis

Refer to the calculation of results section. 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 and Quality Control

Procedures

Reliable laboratory data is critical for making sound environmental decisions. A good quality assurance and 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:

- Defining data quality objectives.
- Choosing analytical methods which will meet those objectives.
- Estimating within-lab precision and bias.
- Setting up control charts.
- Participating 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**, or signs of unsatisfactory organism sensitivity or test performance.

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 that 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, measure concentrations periodically.

Calculation of Results

The bioassay results are calculated using the statistical method described below; two examples are given. Within a 90% 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, use the appropriate t test 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).[6]

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 (α) 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% (EPA, 2002).

[6] https://app.leg.wa.gov/WAC/default.aspx?cite=173-303-100

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) 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 (sT2 + sC2) = 0, H_o is rejected when (pTm - pCm) < 0.5 and H_o is accepted when (pTm - pCm) ≥ 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 3 and 4 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 above.

Table 3: Critical F values with two-tailed alpha (α) = 0.05.

Denominator df	Numerator df 2	Numerator df 3	Numerator df 4	Numerator df 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 4: Critical t values with one-tailed α = 0.10.

df	t[a]	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

Notes:

- a: 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.

There are 10 fish per treatment or control:

- One treatment exposes fish to 10 mg/L of waste using the 80-12 bioassay procedure to determine if the waste sample designates as extremely hazardous waste.
- One treatment exposes fish to 100 mg/L of waste using the 80-12 bioassay procedure to determine if the waste sample designates as dangerous waste.
- The control group is exposed to dilution water only.

Table 5: Example 1 test results.

Replicate (N = 3)	Dilution water control (n = 10)	10 mg/L sample (n = 10)	100 mg/L sample (n = 10)
A: Number dead	1	1	4
A: Proportion dead (p)	0.100	0.100	0.400
B: Number dead	1	2	6
B: Proportion dead (p)	0.100	0.200	0.600
C: Number dead	0	1	5
C: Proportion dead (p)	0	0.100	0.500

Mean (p_m):

- Dilution water control: 0.067
- 10 mg/L sample: 0.133
- 100 mg/L sample: 0.500

Variance (s^2)*:

- Dilution water control: 0.0033
- 10 mg/L sample: 0.0033
- 100 mg/L sample: 0.0100

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 3 and 4, respectively.

*Variance equation is $s^2=\Sigma(p-pm)^2/N-1$ where p = p_C or p_T.

Table 6: 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 3 under Critical F and t values)	39.0	39.0
Calculated t[a] statistic	-9.25	-1.01
Critical t df[b] = [2 (N - 1)]	4	4
Critical t (see Table 4 under Critical F and t values)	-1.53	-1.53
Does waste designate?	No	Yes
Waste Code	Not applicable	WT02

Notes:

- a: 10 mg/L calculated t = $(0.133-0.067-0.5)/[(0.0033+0.0033)/3]^{0.5}$ and 100 mg/L calculated t = $(0.5-0.067-0.5)/[(0.0033+0.0100)/3]^{0.5}$
- b: 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.

There are 15 fish per treatment or control:

- One treatment exposes fish to 10 mg/L of waste using the 80-12 bioassay procedure to determine if the waste sample designates as extremely hazardous waste.
- One treatment exposes fish to 100 mg/L of waste using the 80-12 bioassay procedure to determine if the waste sample designates as dangerous waste.
- The control group is exposed to dilution water only.

Table 7: Example 2 test results.

Replicate (N = 4)	Dilution water control (n = 15)	10 mg/L sample (n = 15)	100 mg/L sample (n = 15)
A: Number dead	0	4	9
A: Proportion dead (p)	0	0.267	0.600
B: Number dead	0	6	8
B: Proportion dead (p)	0	0.400	0.533
C: Number dead	0	3	10
C: Proportion dead (p)	0	0.200	0.667
D: Number dead	0	0	6
D: Proportion dead (p)	0	0	0.400

Mean (p_m):

- Dilution water control: 0
- 10 mg/L sample: 0.217
- 100 mg/L sample: 0.550

Variance (s^2)*:

- Dilution water control: 0
- 10 mg/L sample: 0.0278
- 100 mg/L sample: 0.0130

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 3 and 4 respectively.

*Variance equation is $s^2=\Sigma(p-pm)^2/N-1$ where p = p_C or p_T.

Table 8: 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.	The variances are considered statistically unequal because the control variance is zero. Therefore, the modified t test is used.
Calculated t[a] statistic	-3.39	0.88
Critical t df[b] = (N - 1)	3	3
Critical t (see Table 4 under Critical F and t values)	-1.64	-1.64
Does waste designate?	No	Yes
Waste code	Not applicable	WT02

Notes:

- a: 10 mg/L calculated t = $(0.217-0-0.5)/[(0+0.0278)/4]^{0.5}$ and 100 mg/L calculated t = $(0.550-0-0.5)/[(0+0.0130)/4]^{0.5}$
- b: (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:

- 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, and known physical and chemical properties.
- Dilution water source, chemical characteristics, description of pretreatment, and any additives.
- Detailed information about test organisms. This includes scientific name and how it was verified, wet weight, standard length for fish, age, life stage, source, history, observed diseases, treatments, acclimation procedure, and food used.
- Description of experimental design and test chambers, test solution volume depth in chambers, number of fish per replicate and treatment, loading, and lighting description.
- Description of any aeration performed before or during test.

- Definition of criteria used to determine endpoints, effects, and 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 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.

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Appendix A. 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, check the dilution water for the materials included in Table 9, 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 9: Water quality parameters.*

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°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

*Adapted from Wedemeyer et al. (1981) and EPA (2002).

Appendix B. Dissolved Oxygen

Table 10: Approximate saturation 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

*Based on Pillard (1996).

Appendix C. 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 of water.

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 [GPM] of inflow), to promote the health and quality of salmonids used in toxicity tests.

Table 11: Recommended fish loadings.

Species	Water Temperature (°C)	Water Temperature (°F)	Pounds Fish per GPM[a]: 1000	Pounds Fish per GPM[a]: 500	Pounds Fish per GPM[a]: 100
Coho Salmon	3	38	3.5	5.0	8.0
Coho Salmon	9	48	2.7	4.0	6.0
Coho Salmon	14	58	2.2	3.0	4.5
Coho Salmon	17	63	N/A	2.0	3.5
Fall or Spring Chinook Salmon	3	38	3.0	4.0	6.0
Fall or Spring Chinook Salmon	9	48	2.5	3.0	5.0
Fall or Spring Chinook Salmon	14	58	2.0	2.2	3.5
Fall or Spring Chinook Salmon	17	63	N/A	1.2	3.0

Notes:

- *Adapted from Wedemeyer & Wood (1977).
- a: Fish size (number of fish per pound).

Appendix D. 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 foot-candle).
- **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:** Soft or moderately hard water; see Table 1 in dilution water section.
- **Fish age:** Juvenile fish < 3.0 grams and old enough to be actively feeding.
- **Temperature:** 12 °C ± 1°C.
- **Oxygen/aeration:** None unless DO in danger of dropping below 60% saturation at altitude; ideal DO is 60–100% saturation. If aeration required, all vessels—including controls—should receive the same aeration treatment.
- **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% survival or greater in controls.
- **Reference toxicant:** $CuSo_4$, $CdC1_2$, KCl, NaCl, or ZnSO4 (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:** Soft or moderately hard water >40 mg/L CaCO3 hardness.
- **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 ± 2 rpm) for 18 hours ± 2 hours at 23°C ± 2°C.

Appendix E. Glossary

Term	Meaning
°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
°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

Term	Meaning
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

Appendix F. Terminology

Grammatical terms

Can: Is/are able to.

May: Is/are allowed to.

Might: Could possibly; never used as a synonym for **may** or **can**.

Must: Expresses an absolute requirement.

Should: 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 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: 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: Dangerous and mixed wastes designated per WAC 173-303-100 as extremely hazardous. Also 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% 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 has percolated through or drained from dangerous waste.

Lethal: 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 ion 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: 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.