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# Method 1001.0: Fathead Minnow, *Pimephales promelas,* Larval Survival and Teratogenicity Test; Chronic Toxicity

## Excerpt from:

## Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

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## SECTION 12

## TEST METHOD

## FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST METHOD 1001.0

### 12.1 SCOPE AND APPLICATION

12.1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow, *Pimephales promelas*, using embryos in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

12.1.2 Daily observations on mortality make it possible to also calculate the acute toxicity for desired exposure periods (i.e., 24-h, 48-h, 96-h LC50s).

12.1.3 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

12.1.4 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly degradable and highly volatile toxicants, in the source may not be detected in the test.

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

### 12.2 SUMMARY OF METHOD

12.2.1 Fathead minnow, *Pimephales promelas*, embryos are exposed in a static renewal system to different concentrations of effluent or to receiving water for seven days, starting shortly after fertilization of the eggs. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

### 12.3 INTERFERENCES

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

12.3.2 Adverse effects of low dissolved oxygen (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the presence of toxic substances.

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

12.3.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival and confound test results.

12.3.5 pH drift during the test may contribute to artifactual toxicity when ammonia or other pH-dependent toxicants (such as metals) are present. As pH increases, the toxicity of ammonia also increases (see Subsection 8.8.6), so upward pH drift may increase sample toxicity. For metals, toxicity may increase or decrease with

increasing pH. Lead and copper were found to be more acutely toxic at pH 6.5 than at pH 8.0 or 8.5, while nickel and zinc were more toxic at pH 8.5 than at pH 6.5 (USEPA, 1992). In situations where sample toxicity is confirmed to be artifactual and due to pH drift (as determined by parallel testing as described in Subsection 12.3.5.1), the regulatory authority may allow for control of sample pH during testing using procedures outlined in Subsection 12.3.5.2. It should be noted that artifactual toxicity due to pH drift is not likely to occur unless pH drift is large (more than 1 pH unit) and/or the concentration of some pH-dependent toxicant in the sample is near the threshold for toxicity.

12.3.5.1 To confirm that toxicity is artifactual and due to pH drift, parallel tests must be conducted, one with controlled pH and one with uncontrolled pH. In the uncontrolled-pH treatment, the pH is allowed to drift during the test. In the controlled-pH treatment, the pH is maintained using the procedures described in Subsection 12.3.5.2. The pH to be maintained in the controlled-pH treatment (or target pH) will depend on the objective of the test. If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, the pH should be maintained at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, the pH should be maintained at the pH of the sample upon completion of collection (as measured on an aliquot removed from the sample container).

12.3.5.1.1 During parallel testing, the pH must be measured in each treatment at the beginning (i.e., initial pH) and end (i.e., final pH) of each 24-h exposure period. For each treatment, the mean initial pH (e.g., averaging the initial pH measured each day for a given treatment) and the mean final pH (e.g., averaging the final pH measured each day for a given treatment) must be reported. pH measurements taken during the test must confirm that pH was effectively maintained at the target pH in the controlled-pH treatment. For each treatment, the mean initial pH and the mean final pH should be within ±0.2 pH units of the target pH. Test procedures for conducting toxicity identification evaluations (TIEs) also recommend maintaining pH within ± 0.2 pH units in pH-controlled tests (USEPA, 1992).

12.3.5.1.2 Total ammonia also should be measured in each treatment at the outset of parallel testing. Total ammonia concentrations greater than 5 mg/L in the 100% effluent are an indicator that toxicity observed in the test may be due to ammonia (USEPA, 1992).

12.3.5.1.3 Results from both of the parallel tests (pH-controlled and uncontrolled treatments) must be reported to the regulatory authority. If the uncontrolled test meets test acceptability criteria and shows no toxicity at the permitted instream waste concentration, then the results from this test should be used for determining compliance. If the uncontrolled test shows toxicity at the permitted instream waste concentration, then the results from the pH-controlled test should be used for determining compliance, provided that this test meets test acceptability criteria and pH was properly controlled (see Subsection 12.3.6.1.1).

12.3.5.1.4 To confirm that toxicity observed in the uncontrolled test was artifactual and due to pH drift, the results of the controlled and uncontrolled-pH tests are compared. If toxicity is removed or reduced in the pH-controlled treatment, artifactual toxicity due to pH drift is confirmed for the sample. To demonstrate that a sample result of artifactual toxicity is representative of a given effluent, the regulatory authority may require additional information or additional parallel testing before pH control (as described in Subsection 12.3.5.2) is applied routinely to subsequent testing of the effluent.

12.3.5.2 The pH can be controlled with the addition of acids and bases and/or the use of a $CO_2$-controlled atmosphere over the test chambers. pH is adjusted with acids and bases by dropwise adding 1N NaOH or 1N HCl (see Subsection 8.8.8). The addition of acids and bases should be minimized to reduce the amount of additional ions (Na or Cl) added to the sample. pH is then controlled using the $CO_2$-controlled atmosphere technique. This may be accomplished by placing test solutions and test organisms in closed headspace test chambers, and then injecting a predetermined volume of $CO_2$ into the headspace of each test chamber (USEPA, 1991b; USEPA, 1992); or by placing test chambers in an atmosphere flushed with a predetermined mixture of $CO_2$ and air (USEPA, 1996). Prior experimentation will be needed to determine the appropriate $CO_2$/air ratio or the appropriate volume of $CO_2$ to inject. This volume will depend upon the sample pH, sample volume, container volume, and sample constituents.

If more than 5% $CO_2$ is needed, adjust the solutions with acids (1N HCl) and then flush the headspace with no more than 5% $CO_2$ (USEPA, 1992). If the objective of the WET test is to determine the toxicity of the effluent in the receiving water, $CO_2$ is injected to maintain the test pH at the pH of the receiving water (measured at the edge of the regulatory mixing zone). If the objective of the WET test is to determine the absolute toxicity of the effluent, $CO_2$ is injected to maintain the test pH at the pH of the sample upon completion of collection. USEPA (1991b; 1992) and Mount and Mount (1992) provide techniques and guidance for controlling test pH using a $CO_2$-controlled atmosphere. In pH-controlled testing, control treatments must be subjected to all manipulations that sample treatments are subjected to. These manipulations must be shown to cause no lethal or sublethal effects on control organisms. In pH-controlled testing, the pH also must be measured in each treatment at the beginning and end of each 24-h exposure period to confirm that pH was effectively controlled at the target pH level.

## 12.4 **SAFETY**

12.4.1 See Section 3, Health and Safety.

## 12.5 **APPARATUS AND EQUIPMENT**

12.5.1 Fathead minnow and brine shrimp culture units -- See Section 11, Fathead Minnow, *Pimephales Promelas*, Larval Survival and Growth Test, and USEPA, 2002a. To test effluent toxicity on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from a laboratory fathead minnow culture unit. If necessary, embryos can be shipped in well oxygenated water in insulated containers. In cases where shipping is necessary, up to 48-h old embryos may be used for the test.

12.5.2 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L or more.

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

12.5.4 Environmental chamber or equivalent facility with temperature control ($25 \pm 1°C$).

12.5.5 Water purification system -- MILLIPORE MILLI-Q®, deionized water or equivalent (see Section 5, Facilities, Equipment, and Supplies).

12.5.6 Balance -- analytical, capable of accurately weighing to 0.00001 g.

12.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of material to be weighed.

12.5.8 Test chambers -- four borosilicate glass or disposable, non-toxic plastic labware, per test solution, such as: 500-mL beakers; 100 mm x 15 mm or 100 mm x 20 mm glass or disposable polystyrene Petri dishes; or 12-cm OD, stackable "Carolina" culture dishes. The chambers should be covered with safety glass plates or sheet plastic during the test to avoid potential contamination from the air and excessive evaporation of the test solutions during the test.

12.5.9 Dissecting microscope, or long focal length magnifying lens, hand or stand supported -- for examining embryos and larvae in the test chambers.

12.5.10 Light box, microscope lamp, or flashlight -- for illuminating chambers during examination and observation of embryos and larvae.

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

12.5.12 Volumetric pipets -- Class A, 1-100 mL.

12.5.13 Serological pipets -- 1-10 mL, graduated.

12.5.14 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

12.5.15 Droppers, and glass tubing with fire polished edges, 2-mm ID -- for transferring embryos, and 4-mm ID -- for transferring larvae.

12.5.16 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

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

12.5.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

12.5.19 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA 1979b) -- to calibrate laboratory thermometers.

12.5.20 Meters, pH, DO, and specific conductivity -- for routine physical and chemical measurements.

## 12.6 REAGENTS AND CONSUMABLE MATERIALS

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

12.6.2 Data sheets (one set per test) -- for recording data.

12.6.3 Tape, colored -- for labelling test chambers.

12.6.4 Markers, waterproof -- for marking containers, etc.

12.6.5 Reagents for hardness and alkalinity tests -- see USEPA Methods 130.2 and 310.1, USEPA 1979b.

12.6.6 Membranes and filling solutions for DO probe (see USEPA Method 360.1, USEPA 1979b), or reagents -- for modified Winkler analysis.

12.6.7 Standard pH buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for instrument calibration (see USEPA Method 150.1, USEPA 1979b).

12.6.8 Specific conductivity standards -- see USEPA Method 120.1, USEPA 1979b.

12.6.9 Laboratory quality control samples and standards -- for calibration of the above methods.

12.6.10 Reference toxicant solutions -- see Section 4, Quality Assurance.

12.6.11 Reagent water -- defined as distilled or deionized water which does not contain substances which are toxic to the test organisms (see Section 5, Facilities, Equipment, and Supplies).

12.6.12 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water; and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

12.6.13 TEST ORGANISMS, FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

12.6.13.1 Fathead minnow embryos, less than 36-h old, are used for the test. The test is conducted with four (minimum of three) test chambers at each toxicant concentration and control. Fifteen (minimum of ten) embryos are placed in each replicate test chamber. Thus 60 (minimum of 30) embryos are exposed at each test concentration and 360 (minimum of 180) embryos would be needed for a test consisting of five effluent concentrations and a control.

12.6.13.2 Sources of Organisms

12.6.13.2.1 It is recommended that the embryos be obtained from inhouse cultures or other local sources if at all possible, because it is often difficult to ship the embryos so that they will be less than 36 h old for beginning the test. Receipt of embryos via Express Mail, air express, or other carrier, from a reliable outside source is an acceptable alternative, but they must not be over 48 h old when used to begin the test.

12.6.13.2.2 Culturing methods for fathead minnows, *Pimephales promelas*, are described in Section 6, Section 11 and in USEPA, 2002a.

12.6.13.2.3 Fish obtained from outside sources (see Section 5, Facilities, Equipment, and Supplies) such as commercial biological supply houses for use as brood stock should be guaranteed to be (1) of the correct species, (2) disease free, (3) in the requested age range, and (4) in good condition. This can be done by providing the record of the date on which the eggs were laid and hatched, and information on the sensitivity of the contemporary fish to reference toxicants.

12.6.13.3 Obtaining Embryos for Toxicity Tests from Inhouse Cultures.

12.6.13.3.1 Spawning substrates with the newly-spawned, fertilized embryos are removed from the spawning tanks or ponds, and the embryos are separated from the spawning substrate by using the index finger and rolling the embryos gently with a circular movement of the finger (see Gast and Brungs, 1973). The embryos are then combined and washed from the spawning substrate onto a 400 µm NITEX® screen, sprayed with a stream of deionized water to remove detritus and food particles, and back-washed with dilution water into a crystallizing dish for microscopic examination. Damaged and infertile eggs are discarded.

12.6.13.3.2 The embryos from three or more spawns are pooled in a single container to provide a sufficient number to conduct the tests. These embryos may be used immediately to start a test inhouse or may be transported for use at a remote location. When transportation is required, embryos should be taken from the substrates within 12 h of spawning. This permits off-site tests to be started with less than 36-h old embryos. Embryos should be transported or shipped in clean, opaque, insulated containers, in well aerated or oxygenated fresh culture or dilution water, and should be protected from extremes of temperature and any other stressful conditions during transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution water, should be less than 2°C. Sudden changes in pH, dissolved ions, osmotic strength, and DO should be avoided.

## 12.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

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

## 12.8 CALIBRATION AND STANDARDIZATION

12.8.1 See Section 4, Quality Assurance.

12.9 **QUALITY CONTROL**

12.9.1 See Section 4, Quality Assurance.

12.10 **TEST PROCEDURES**

12.10.1 TEST SOLUTIONS

12.10.1.1 Receiving Waters

12.10.1.1.1 The sampling point is determined by the objectives of the test. Receiving water toxicity is determined with samples used directly as collected or after samples are passed through a 60 µm NITEX® filter and compared without dilution, against a control. Using four replicate chambers per test, each containing 100 mL, and 400 mL for chemical analysis, would require approximately one liter, or more, of sample per test day.

12.10.1.2 Effluents

12.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Improvements in precision decline rapidly if the dilution factor is increased beyond 0.5 and precision declines rapidly if a smaller dilution factor is used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.**

12.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions should be added at the lower range of effluent concentrations.

12.10.1.2.3 The volume of effluent required for daily renewal of four replicates per concentration, each containing 100 mL of test solution, is 1.5 L. Sufficient test solution (approximately 1000 mL) is prepared at each effluent concentration to provide 400 mL additional volume for chemical analyses. If the sample is used for more than one daily renewal of test solutions, the volume must be increased proportionately.

12.10.1.2.4 Tests should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for the off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

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

12.10.1.2.6 The DO of the test solutions should be checked prior to test initiation. If any of the solutions are supersaturated with oxygen, all of the solutions and the control should be gently aerated. If any solution has a DO below 4.0 mg/L, all of the solutions and the control must be gently aerated.

12.10.1.3 Dilution Water

12.10.1.3.1 Dilution water may be uncontaminated receiving water, a standard synthetic (reconstituted) water, or some other uncontaminated natural water (see Section 7, Dilution Water).

12.10.1.3.2 If the hardness of the test solutions (including the control) does not equal or exceed 25 mg/L as $CaCO_3$, it may be necessary to adjust the hardness by adding reagents for synthetic softwater as listed in Table 3, Section 7. In this case parallel tests should be conducted, one with the hardness adjusted and one unadjusted.

12.10.2 START OF THE TEST

12.10.2.1 Label the test chambers with a marking pen and use color-coded tape to identify each treatment and replicate. A minimum of five effluent concentrations and a control are used for each effluent test. Each treatment (including the control) should have four (minimum of three) replicates.

12.10.2.2 Tests performed in laboratories that have inhouse fathead minnow breeding cultures must initiate tests with embryos less than 36 h old. When the embryos must be shipped to the test site from a remote location, it may be necessary to use embryos older than 36 h because of the difficulty of coordinating test organism shipments with field operations. However, in the latter case, the embryos must not be more than 48 h old at the start of the test and should all be within 24 h of the same age.

12.10.2.3 Randomize the position of the test chambers at the beginning of the test (see Appendix A). Maintain the chambers in this configuration throughout the test. Preparation of a position chart may be helpful.

12.10.2.4 The test organisms should come from a pool of embryos consisting of at least three separate spawnings. Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are thoroughly mixed.

12.10.2.5 Using a small bore (2 mm ID) glass tube, the embryos are placed one or two at a time into each randomly arranged test chamber or intermediate container in sequential order, until each chamber contains 15 (minimum of 10) embryos, for a total of 60 (minimum of 30) embryos for each concentration (see Appendix A). The amount of water added to the chambers when transferring the embryos to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

12.10.2.6 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with new undamaged embryos. Placing the test chambers on a light table may facilitate examining and counting the embryos.

12.10.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

12.10.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 $\mu E/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at $25 \pm 1\,°C$.

12.10.4 DISSOLVED OXYGEN (DO) CONCENTRATION

12.10.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should be measured in the new solutions at the start of the test (Day 0) and before daily renewal of the new solutions on subsequent days. The DO concentrations should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a l-mL KIMAX® serological Pipet, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the embryos.

12.10.5 FEEDING

12.10.5.1 Feeding is not required.

12.10.6 OBSERVATIONS DURING THE TEST

12.10.6.1 Routine Chemical and Physical Determinations

12.10.6.1.1 DO is measured at the beginning and end of each 24-h exposure period in at least one test chamber at each test concentrations and in the control.

12.10.6.1.2 Temperature and pH are measured at the end of each 24-h exposure period in at least one test chamber at each test concentration and in the control. Temperature should also be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test vessels, at least at the end of the test, to determine temperature variation in the environmental chamber.

12.10.6.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

12.10.6.1.4 Conductivity, alkalinity and hardness are measured in each new sample (100% effluent or receiving water) and in the control.

12.10.6.2 Record all the measurements on the data sheet (Figure 1).

12.10.6.3 Routine Biological Observations

12.10.6.3.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine the embryos. Remove the dead embryos (milky colored and opaque) and record the number (Figure 2). If the rate of mortality (including those with fungal infection) exceeds 20% in the control chambers, or if excessive non-concentration-related mortality occurs, terminate the test and start a new test with new embryos.

12.10.6.3.2 At 25°C, hatching may begin on the fourth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed larvae, daily. Deformed larvae are those with gross morphological abnormalities such as lack of appendages, lack of fusiform shape (non-distinct mass), lack of mobility, a colored, beating heart in an opaque mass, or other characteristics that preclude survival. Count and remove dead embryos and larvae as previously discussed and record the numbers for all of the test observations (Figure 2). Upon hatching, deformed larvae are counted as dead.

12.10.6.3.3 Protect the embryos and larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead organisms carefully. Make sure that the test organisms remain immersed during the performance of the above operations.

12.10.7 DAILY CLEANING OF TEST CHAMBERS

12.10.7.1 Since feeding is not required, test chambers are not cleaned daily unless accumulation of particulate matter at the bottom of the chambers causes a problem.

12.10.8 TEST SOLUTION RENEWAL

12.10.8.1 Freshly prepared solutions are used to renew the tests daily. For on-site toxicity studies, fresh effluent or receiving water samples should be collected daily, and no more than 24 h should elapse between collection of the samples and their use in the tests (see Section 8, Effluent and Receiving Water Sampling, Sample Handling and Sample Preparation for Toxicity Tests). For off-site tests, a minimum of three samples are collected, preferably on days one, three, and five. Maintain the samples in the refrigerator at 0-6°C until used.

12.10.8.2 The test solutions are renewed immediately after removing dead embryos and/or larvae. During the daily renewal process, the water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence and possible injury to the embryos or larvae.

Discharger:____________________ Test Dates:____________________

Location:____________________ Analyst:____________________

| | Day | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Control: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

| | Day | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Conc: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

| | Day | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Conc: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations.

Discharger:______________________ Test Dates:______________________

Location:______________________ Analyst:______________________

| Control: | Day 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
|---|---|---|---|---|---|---|---|---|
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

| Conc: | Day 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
|---|---|---|---|---|---|---|---|---|
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

| Conc: | Day 1 | 2 | 3 | 4 | 5 | 6 | 7 | Remarks |
|---|---|---|---|---|---|---|---|---|
| Temp. | | | | | | | | |
| D.O. Initial | | | | | | | | |
| Final | | | | | | | | |
| pH Initial | | | | | | | | |
| Final | | | | | | | | |
| Alkalinity | | | | | | | | |
| Hardness | | | | | | | | |
| Conductivity | | | | | | | | |
| Chlorine | | | | | | | | |

Figure 1. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Routine chemical and physical determinations (CONTINUED)

Discharger:______________________________ Test Dates:__________________________

Location:________________________________ Analyst:____________________________

______________________________________________________________________

| Conc: | Rep. No. | Condition of Embryo/larvae | Day | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Control | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| Treatment | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| Treatment | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| Treatment | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data.

Discharger:________________________________ Test Dates:____________________________

Location:_________________________________ Analyst:______________________________

___________________________________________________________________________

| Conc: | Rep. No. | Condition of Embryo/larvae | Day | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Treatment | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| Treatment | 1 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 2 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 3 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |
| | 4 | Live/dead | | | | | | | |
| | | Terata | | | | | | | |

Comments:

Figure 2. Data form for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test. Survival and terata data (CONTINUED).

12.10.9 TERMINATION OF THE TEST

12.10.9.1 The test is terminated after seven days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each (Figure 2). The deformed larvae are treated as dead in the analysis of the data. Keep a separate record of the total number and percent of deformed larvae for use in reporting the teratogenicity of the test solution.

12.10.9.2 Prepare a summary of the data as illustrated in Figure 3.

## 12.11 **SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA**

12.11.1 A summary of test conditions and test acceptability criteria is presented in Table 1.

## 12.12 **ACCEPTABILITY OF TEST RESULTS**

12.12.1 For the test results to be acceptable, survival in the controls must be at least 80%.

Discharger: ______________________ Test Dates: ______________________

Location: ______________________ Analyst: ______________________

| Treatment | Control | | | | | |
|---|---|---|---|---|---|---|
| No. dead embryos and larvae | | | | | | |
| No. terata | | | | | | |
| Total mortality (dead and deformed) | | | | | | |
| Total mortality (%) | | | | | | |
| Terata (%) | | | | | | |
| Hatch (%) | | | | | | |

Comments:

Figure 3. Summary data for the fathead minnow, *Pimephales promelas*, embryo-larval survival and teratogenicity test.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0)[1]

| | |
|---|---|
| 1. Test type: | Static renewal (required) |
| 2. Temperature: | 25 ± 1°C (recommended)<br>Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required) |
| 3. Light quality: | Ambient laboratory illumination (recommended) |
| 4. Light intensity: | 10-20 µE/$m^2$/s or 50-100 ft-c (ambient laboratory levels) (recommended) |
| 5. Photoperiod: | 16 h light, 8 h dark (recommended) |
| 6. Test chamber size: | 150 mL (recommended minimum) |
| 7. Test solution volume: | 70 mL (recommended minimum) |
| 8. Renewal of test solutions: | Daily (required) |
| 9. Age of test organisms: | Less than 36-h old embryos (Maximum of 48-h if shipped) (required) |
| 10. No. embryos per test chamber: | 15 (recommended)<br>10 (required minimum) |
| 11. No. replicate test chambers per concentration: | 4 (recommended)<br>3 (required minimum) |
| 12. No. embryos per concentration: | 60 (recommended)<br>30 (required minimum) |
| 13. Feeding regime: | Feeding not required |
| 14. Aeration: | None unless DO falls below 4.0 mg/L (recommended) |

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 1. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (TEST METHOD 1001.0) (CONTINUED)

| | |
|---|---|
| 15. Dilution water: | Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or DMW (see Section 7, Dilution Water). The hardness of the test solutions should equal or exceed 25 mg/L ($CaCO_3$) to ensure hatching success (available options) |
| 16. Test concentrations: | Effluents: 5 and a control (required minimum)<br>Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended) |
| 17. Dilution factor: | Effluents: ≥ 0.5 (recommended)<br>Receiving waters: None, or ≥ 0.5 (recommended) |
| 18. Test duration: | 7 days (required) |
| 19. Endpoint: | Combined mortality (dead and deformed organisms) (required) |
| 20. Test acceptability criteria: | 80% or greater survival in controls (required) |
| 21. Sampling requirements: | For on-site tests, samples collected daily and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples (e.g., collected on days one, three, and five) with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required) |
| 22. Sample volume required: | 1.5 to 2.5 L/day depending on the volume of test solutions used (recommended) |

## 12.13 **DATA ANALYSIS**

### 12.13.1 GENERAL

12.13.1.1 Tabulate and summarize the data (Figure 3).

12.13.1.2 The endpoints of this toxicity test are based on total mortality, combined number of dead embryos, and dead and deformed larvae. The EC1 is calculated using Probit Analysis (Finney, 1971; see Appendix I). Separate analyses are performed for the estimation of LOEC and NOEC endpoints and for the estimation of the EC1 endpoint. Concentrations at which there is no survival in any of the test chambers are excluded from the statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint. See the Appendices for examples of the manual computations and examples of data input and output for the computer programs.

12.13.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

### 12.13.2 EXAMPLE OF ANALYSIS OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY DATA

12.13.2.1 Formal statistical analysis of the total mortality data is outlined on the flowchart in Figure 4. The response used in the analysis is the total mortality proportion in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the EC endpoint. Concentrations at which there is 100% total mortality in all of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the EC1 endpoint.

12.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

12.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative (see Appendix F).

12.13.2.4 Probit Analysis (Finney, 1971) is used to estimate the concentration that causes a specified percent decrease in survival from the control. In this analysis, the total mortality data from all test replicates at a given concentration are combined.

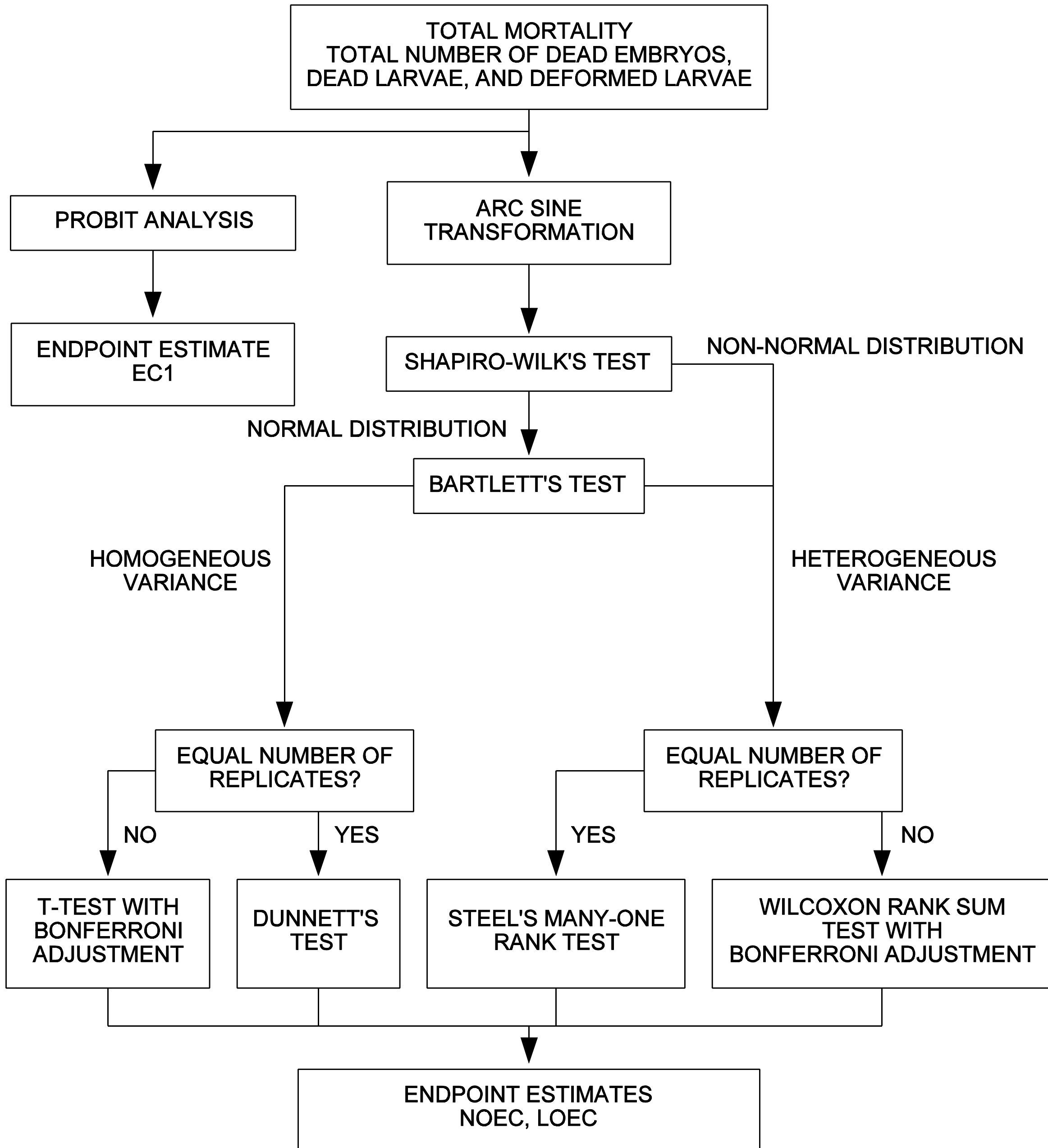


Figure 4. Flowchart for statistical analysis of fathead minnow, *Pimephales promelas*, embryo-larval data.

12.13.2.5 The data for this example are listed in Table 2. Total mortality, expressed as a proportion (combined total number of dead embryos, dead larvae and deformed larvae divided by the number of embryos at start of test), is the response of interest. The total mortality proportion in each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each effluent concentration and control are listed in Table 3. A plot of the data is provided in Figure 5. Since there is 100% total mortality in replicates for the 50.0% concentration, it is not included in this statistical analysis and is considered a qualitative mortality effect.

TABLE 2. DATA FROM FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL TOXICITY TEST WITH GROUND WATER EFFLUENT

| Effluent Conc. (%) | No. Eggs at Start | Dead at Test Termination | | Deformed at Test Termination | | Dead + Deformed at Termination | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Control | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 2 | 20 | 0 | 0 | 2 | 20 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 1 | 10 | 0 | 0 | 1 | 10 |
| 3.125 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 1 | 10 | 1 | 10 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 1 | 10 | 0 | 0 | 1 | 10 |
| 6.25 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 1 | 10 | 1 | 10 |
| 12.5 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 1 | 10 | 0 | 0 | 1 | 10 |
| 25.0 | 10 | 1 | 10 | 9 | 90 | 10 | 100 |
| | 10 | 2 | 20 | 8 | 80 | 10 | 100 |
| | 10 | 2 | 20 | 8 | 80 | 10 | 100 |
| | 10 | 1 | 10 | 4 | 40 | 5 | 50 |
| 50.0 | 10 | 4 | 40 | 6 | 60 | 10 | 100 |
| | 10 | 3 | 30 | 7 | 70 | 10 | 100 |
| | 10 | 5 | 50 | 5 | 50 | 10 | 100 |
| | 10 | 3 | 30 | 7 | 70 | 10 | 100 |

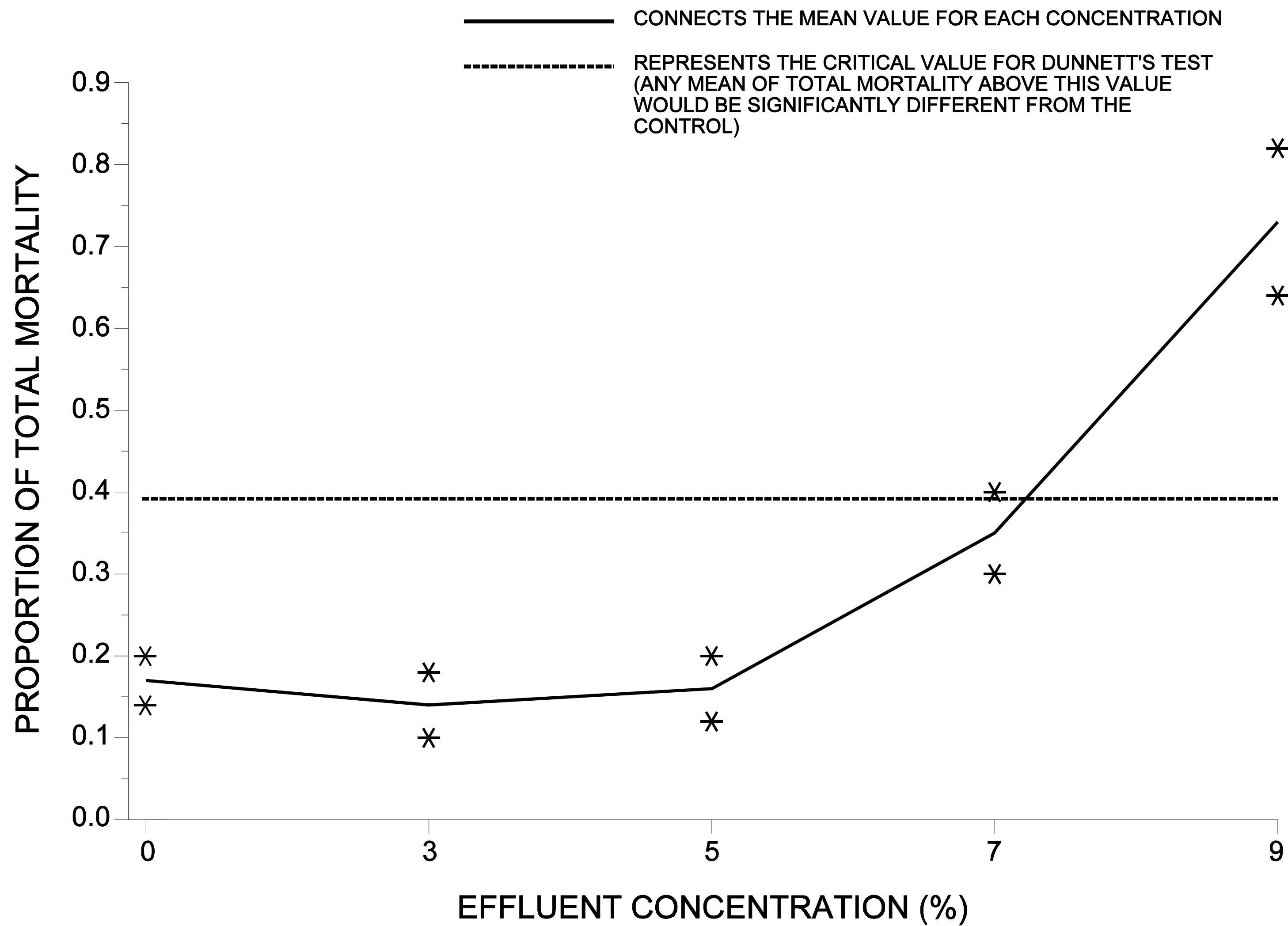


Figure 5. Plot of fathead minnow, *Pimephales promelas*, total mortality data from the embryo-larval test.

TABLE 3. FATHEAD MINNOW, *PIMEPHALES PROMELAS,* EMBRYO-LARVAL TOTAL MORTALITY DATA

| | Replicate | Control | Effluent Concentration (%) 3.125 | 6.25 | 12.5 | 25.0 | 50.0 |
|---|---|---|---|---|---|---|---|
| RAW | A | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 |
| | B | 0.20 | 0.10 | 0.00 | 0.00 | 1.00 | 1.00 |
| | C | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 |
| | D | 0.10 | 0.10 | 0.10 | 0.10 | 0.50 | 1.00 |
| ARC SINE TRANS-FORMED | A | 0.159 | 0.159 | 0.159 | 0.159 | 1.412 | - |
| | B | 0.464 | 0.322 | 0.159 | 0.159 | 1.412 | - |
| | C | 0.159 | 0.159 | 0.159 | 0.159 | 1.412 | - |
| | D | 0.322 | 0.322 | 0.322 | 0.322 | 0.785 | - |
| Mean($\bar{Y}_i$) | | 0.276 | 0.241 | 0.200 | 0.200 | 1.255 | |
| $S_i^2$ | | 0.022 | 0.009 | 0.007 | 0.007 | 0.098 | |
| i | | 1 | 2 | 3 | 4 | 5 | |

12.13.2.6 Test for Normality

12.13.2.6.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 4.

TABLE 4. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

| Replicate | Control | Effluent Concentration (%) 3.125 | 6.25 | 12.5 | 25.0 | 50.0 |
|---|---|---|---|---|---|---|
| A | -0.117 | -0.082 | -0.041 | -0.041 | 0.157 | - |
| B | 0.188 | 0.081 | -0.041 | -0.041 | 0.157 | - |
| C | -0.117 | 0.081 | -0.041 | -0.041 | 0.157 | - |
| D | 0.046 | -0.082 | 0.122 | 0.122 | -0.470 | - |

12.13.2.6.2 Calculate the denominator, D, of the statistic:

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

Where: $X_i$ = the ith centered observation

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

$n$ = the total number of centered observations

12.13.2.6.3 For this set of data, $n = 20$

$$\bar{X} = \frac{1}{20}(-0.003) = 0.000$$

$$D = 0.4261$$

12.13.2.6.4 Order the centered observations from smallest to largest

$$X^{(1)} \leq X^{(2)} \leq ... \leq X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 5.

TABLE 5. ORDERED CENTERED OBSERVATIONS FOR THE SHAPIRO-WILK'S EXAMPLE

| i | $X^{(i)}$ | i | $X^{(i)}$ |
|---|---|---|---|
| 1 | -0.470 | 11 | -0.041 |
| 2 | -0.117 | 12 | 0.046 |
| 3 | -0.117 | 13 | 0.081 |
| 4 | -0.082 | 14 | 0.081 |
| 5 | -0.082 | 15 | 0.122 |
| 6 | -0.041 | 16 | 0.122 |
| 7 | -0.041 | 17 | 0.157 |
| 8 | -0.041 | 18 | 0.157 |
| 9 | -0.041 | 19 | 0.157 |
| 10 | -0.041 | 20 | 0.188 |

12.13.2.6.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, ..., a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 20 and k = 10. The $a_i$ values are listed in Table 6.

TABLE 6. COEFFICIENTS AND DIFFERENCES FOR THE SHAPIRO-WILK'S EXAMPLE

| i | $a_i$ | $X^{(n-i+1)} - X^{(i)}$ | |
|---|---|---|---|
| 1 | 0.4734 | 0.658 | $X^{(20)} - X^{(1)}$ |
| 2 | 0.3211 | 0.274 | $X^{(19)} - X^{(2)}$ |
| 3 | 0.2565 | 0.274 | $X^{(18)} - X^{(3)}$ |
| 4 | 0.2085 | 0.239 | $X^{(17)} - X^{(4)}$ |
| 5 | 0.1686 | 0.204 | $X^{(16)} - X^{(5)}$ |
| 6 | 0.1334 | 0.163 | $X^{(15)} - X^{(6)}$ |
| 7 | 0.1013 | 0.122 | $X^{(14)} - X^{(7)}$ |
| 8 | 0.0711 | 0.122 | $X^{(13)} - X^{(8)}$ |
| 9 | 0.0422 | 0.087 | $X^{(12)} - X^{(9)}$ |
| 10 | 0.0140 | 0.000 | $X^{(11)} - X^{(10)}$ |

12.13.2.6.6 Compute the test statistic, W, as follows:

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

The differences $X^{(n-i+1)} - X^{(i)}$ are listed in Table 6. For the data in this example,

$$W = \frac{1}{0.4261}(0.6004)^2$$
$$= 0.846$$

12.13.2.6.7 The decision rule for this test is to compare W as calculated in Section 13.2.6.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 20 observations is 0.868. Since W = 0.846 is less than the critical value, conclude that the data are not normally distributed.

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

12.13.2.7 Steel's Many-one Rank Test

12.13.2.7.1 For each control and concentration combination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1, 2, ..., 8) to the ordered observations with a rank of 1 assigned to the smallest observation, rank of 2 assigned to the next larger observation, etc. If ties occur when ranking, assign the average rank to each tied observation.

12.13.2.7.2 An example of assigning ranks to the combined data for the control and 3.125% effluent concentration is given in Table 7. This ranking procedure is repeated for each control/concentration combination. The complete set of rankings is summarized in Table 8. The control group ranks are next summed for each effluent concentration pairing, as shown in Table 9.

TABLE 7. ASSIGNING RANKS TO THE CONTROL AND 3.125% EFFLUENT CONCENTRATION FOR STEEL'S MANY-ONE RANK TEST

| Rank | Transformed Proportion Mortality | Effluent Concentration (%) |
|---|---|---|
| 2.5 | 0.159 | Control |
| 2.5 | 0.159 | Control |
| 2.5 | 0.159 | 3.125 |
| 2.5 | 0.159 | 3.125 |
| 6 | 0.322 | Control |
| 6 | 0.322 | 3.125 |
| 6 | 0.322 | 3.125 |
| 8 | 0.464 | Control |

TABLE 8. TABLE OF RANKS FOR STEEL'S MANY-ONE RANK TEST

| Repl. | Control | | Effluent Concentration (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3.125 | | 6.25 | | 12.5 | | 25.0 | |
| A | 0.159 | (2.5,3,3,1.5) | 0.159 | (2.5) | 0.159 | (3) | 0.159 | (3) | 1.412 | (7) |
| B | 0.464 | (8,8,8,4) | 0.322 | (6) | 0.159 | (3) | 0.159 | (3) | 1.412 | (7) |
| C | 0.159 | (2.5,3,3,1.5) | 0.159 | (2.5) | 0.159 | (3) | 0.159 | (3) | 1.412 | (7) |
| D | 0.322 | (6,6.5,6.5,3) | 0.322 | (6) | 0.322 | (3) | 0.159 | (3) | 0.785 | (5) |

TABLE 9. RANK SUMS

| Effluent Concentration (%) | Control Rank Sum |
|---|---|
| 3.125 | 19 |
| 6.25 | 20.5 |
| 12.5 | 20.5 |
| 25.0 | 10 |

12.13.2.7.3 For this example, we want to determine if the total mortality in any of the effluent concentrations is significantly higher than the total mortality in the control. If this occurs, the rank sum of the control would be significantly less than the rank sum at that concentration. Thus we are only concerned with comparing the control rank sum for each pairing with the various effluent concentrations with some "minimum" or critical rank sum, at or below which the concentration total mortality would be considered significantly greater than the control. At a signficance level of 0.05, the minimum rank sum in a test with four concentrations (excluding the control) and four replicates per concentration is 10 (see Table 5, Appendix E).

12.13.2.7.4 Since the control rank sum for the 25.0% effluent concentration pairing is equal to the critical value, the total proportion mortality in the 25.0% concentration is considered significantly greater than that in the control. Since no other rank sums are less than or equal to the critical value, no other concentrations have signficantly higher total proportion mortality than the control. Hence the NOEC is 12.5% and the LOEC is 25.0%.

12.13.2.8 Calculation of the LC50

12.13.2.8.1 The data used for the Probit Analysis is summarized in Table 10. To perform the Probit Analysis, run the USEPA Probit Analysis Program. An example of the program input and output is supplied in Appendix I.

12.13.2.8.2 For this example, the chi-square test for heterogeneity was not significant. Thus Probit Analysis appears appropriate for this data.

12.13.2.8.3 Figure 6 shows the output data for the Probit Analysis of the data from Table 10 using the USEPA Probit Program.

TABLE 10. DATA FOR PROBIT ANALYSIS

| | | Effluent Concentration (%) | | | | |
|---|---|---|---|---|---|---|
| | Control | 3.125 | 6.25 | 12.5 | 25.0 | 50.0 |
| Number Dead | 3 | 1 | 0 | 1 | 6 | 15 |
| Number Exposed | 40 | 40 | 40 | 40 | 40 | 40 |

## 12.14 **PRECISION AND ACCURACY**

### 12.14.1 PRECISION

12.14.1.1 Single-laboratory Precision

12.14.1.1.1 Data shown in Tables 11 and 12 indicate that the precision of the embryo-larval survival and teratogenicity test, expressed as the relative standard deviation (or coefficient of variation, CV) of the LC1 values, was 62% for cadmium (Table 11) and 41% for Diquat (Table 12).

12.14.1.1.2 Precision data are also available from four embryo-larval survival and teratogenicity tests on trickling filter pilot plant effluent (Table 13). Although the data could not be analyzed by Probit Analysis, the NOECs and LOECs obtained using Dunnett's Procedure were the same for all four tests, 7% and 11% effluent, respectively, indicating maximum precision in terms of the test design.

12.14.1.2 Multilaboratory Precision

12.14.1.2.1 Data on the multilaboratory precision of this test are not yet available.

### 12.14.2 ACCURACY

12.14.2.1 The accuracy of toxicity tests cannot be determined.

USEPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

| Conc. | Number Exposed | Number Resp. | Observed Proportion Responding | Proportion Responding Adjusted for Controls |
|---|---|---|---|---|
| Control | 20 | 2 | 0.1000 | 0.0000 |
| 0.5000 | 20 | 2 | 0.1000 | 0.0174 |
| 1.0000 | 20 | 1 | 0.0500 | -.0372 |
| 2.0000 | 20 | 4 | 0.2000 | 0.1265 |
| 4.0000 | 20 | 16 | 0.8000 | 0.7816 |
| 8.0000 | 20 | 20 | 1.0000 | 1.0000 |

Chi - Square for Heterogeneity (calculated) = 0.441
Chi - Square for Heterogeneity (tabular value) = 7.815

Probit Analysis of Fathead Minnow Embryo-Larval Survival
and Teratogenicity Data

Estimated LC/EC Values and Confidence Limits

| Point | Exposure Conc. | Lower 95% Confidence Limits | Upper |
|---|---|---|---|
| LC/EC 1.00 | 1.346 | 0.453 | 1.922 |
| LC/EC 50.00 | 3.018 | 2.268 | 3.672 |

Figure 6. Output for USEPA Probit Program, Version 1.5.

TABLE 11. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[1,2]

| Test | LC1[3] (mg/L) | 95% Confidence Limits | NOEC[4] (mg/L) |
|---|---|---|---|
| 1 | 0.014 | 0.009 - 0.018 | 0.012 |
| 2 | 0.006 | 0.003 - 0.010 | 0.012 |
| 3 | 0.005 | 0.003 - 0.009 | 0.013 |
| 4 | 0.003 | 0.002 - 0.004 | 0.011 |
| 5 | 0.006 | 0.003 - 0.009 | 0.012 |
| N | 5 | | 5 |
| Mean | 0.0068 | | NA |
| SD | 0.0042 | | |
| CV(%) | 62 | | NA |

[1] Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

[2] Cadmium chloride was used as the reference toxicant. The nominal concentrations, expressed as cadmium (mg/L), were: 0.01, 0.032, 0.100, 0.320, and 1.000. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

[3] Determined by Probit Analysis.

[4] Highest no-observed-effect concentration determined by independent statistical analysis (2X2 Chi-square Fisher's Exact Test). NOEC range of 0.011 - 0.013 represents a difference of one exposure concentration.

TABLE 12. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[1,2]

| Test | LC1[3] (mg/L) | 95% Confidence Limits |
|---|---|---|
| 1 | 0.58 | 0.32 - 0.86 |
| 2 | 2.31 | --[4] |
| 3 | 1.50 | 1.05 - 1.87 |
| 4 | 1.71 | 1.24 - 2.09 |
| 5 | 1.43 | 0.93 - 1.83 |
| N | 5 | |
| Mean | 1.51 | |
| SD | 0.62 | |
| CV(%) | 41.3 | |

1 Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Bioassessment and Ecotoxicology Branch, EMSL, USEPA, Cincinnati, OH.

2 The Diquat concentrations were determined by chemical analysis. The dilution water was reconstituted water with a hardness of 100 mg/L as calcium carbonate, and a pH of 7.8.

3 Determined by Probit Analysis.

4 Cannot be calculated.

TABLE 13. PRECISION OF THE FATHEAD MINNOW, *PIMEPHALES PROMELAS*, EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[1,2,3]

| Test No. | NOEC (% Effluent) | LOEC (% Effluent) |
|---|---|---|
| 1 | 7 | 11 |
| 2 | 7 | 11 |
| 3 | 7 | 11 |
| 4 | 7 | 11 |

[1] Data provided by Timothy Neiheisel, Bioassessment and Ecotoxiology Branch, EMSL, USEPA, Cincinnati, OH.
[2] Effluent concentrations used: 3, 5, 7, 11 and 16%
[3] Maximum precision achieved in terms of NOEC-LOEC interval. For a discusssion of the precision of data from chronic toxicity tests (see Section 4, Quality Assurance).