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# Fathead Minnow Larval Survival and Growth Toxicity Tests

## Supplemental Report For Video Training Tape

# SUPPLEMENTAL REPORT FOR VIDEO TRAINING TAPE ON FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TOXICITY TESTS

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# FOREWORD

The material presented in the video tape and this report is based on the document Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Some of the test conditions, parameters, and methods of this manual are in the process of being revised and were not published at the time of the completion of this project. The methods presented here represent the latest accepted revisions.

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FATHEAD MINNOW LARVAL SURVIVAL AND
GROWTH TOXICITY TEST

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# INTRODUCTION

This report accompanies the Environmental Protection Agency's video training tape for conducting fathead minnow larval survival and growth toxicity tests. The test method is found in Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. And is adapted from methods developed by Teresa Norberg-King and Dr. Donald Mount of EPA's Environmental Research Laboratory, Duluth, Minnesota. The material presented in both the videotape and this report summarzies the methods but does not replace a thorough review and understanding of the methods by laboratory personnel before conducting the test.

# BACKGROUND

Clean Water Act, Section 402

Under the National Pollutant Discharge Elimination System (NPDES) program, EPA uses toxicity tests to monitor and evaluate effluents for their toxicity to biota and their impact on receiving waters. By determining acceptable or safe concentrations for toxicants discharged into receiving waters, EPA can establish NPDES permit limitations for toxicity. These permit limitations regulate pollutant discharges by a whole effluent toxicity approach rather than on a chemical specific basis.

The test method requires a static renewal exposure system. Every 24 hours, the fish are moved to a new tank containing a freshly prepared solution of the appropriate effluent concentration.

The fathead minnow subchronic test is a seven-day static renewal exposure for determining sublethal toxicity in order to estimate chronic toxicity. The test method determines the toxicity of an effluent by exposing larval fathead minnows (Pimephales promelas) to a series of effluent concentrations. The effect of the effluent is measured by the survival and growth of the fish. Minnows that are 24 hours old or less are exposed, and growth is measured as the difference in the fishes' average mean dry weight compared to that of the controls. This report covers the procedures for conducting the seven-day fathead minnow test and also describes some helpful procedures that are not presented in the manual.

## TEST METHOD

Section 8 of the Chronic Methods Manual covers sample collection. Note that surface waters must be filtered (80 um plankton net) for fathead minnow tests.

Effluent sampling should be conducted according to the methods manual. The test should be started on the arrival date of the sample and within 72 hours of sample collection. Warm the effluent to 25 $\pm$ 1°C slowly to avoid exceeding the desired temperature. This is done using a water bath and monitoring the temperature closely. The temperature must be maintained throughout the seven-day test period. Once the effluent and the dilution water have reached the desired temperature, the dilutions can be prepared. Use a minimum of five exposure concentrations and a control with a minimum of three or four replicates per

concentration. The volume of test solution required is 250 ml per replicate.

Routine Chemistries

Once the various concentrations are prepared, set aside one aliquot of each for the routine chemistries that must be performed. By setting these aside, the chemistries can be performed without contaminating the actual test solutions with the probe. For test initiation and renewals, measure and record the dissolved oxygen at the beginning of each 24 hour renewal in each test concentration. This procedure will also be performed at the end of the final exposure period for one replicate in each concentration and the control. Also, the temperature, pH, and conductivity must be measured and recorded at the beginning of each exposure period. Temperature and pH must also be measured and recorded at the end of each period. Alkalinity and hardness are measured and recorded in the control and highest concentration only, at the beginning and end of each 24 hour period. See Table 1.

It is recommended that the temperature be recorded continuously during the test.

The test chamber should not be smaller than a one liter beaker or a glass aquarium that is 7.6 cm wide by 16 cm long by 8 cm high. The surface-to-volume ratio of the beaker and the aquarium is approximately the same. The test chambers should be placed in a temperature and photoperiod controlled room or environment and should be randomized after the test solution is added to each replicate.

Table 1. Monitoring Schedule

| Parameter | Monitoring Frequency | |
|---|---|---|
| | Beginning of 24-hr exposure | End of 24-hr exposure |
| Dissolved Oxygen | X | X[1] |
| Temperature | X | X |
| pH | X | X |
| Conductivity | X | |
| Alkalinity[2] | X | X |
| Hardness[2] | X | X |

[1] End measurement on one replicate in each concentration and the control.

[2] Measure in highest concentration and control only.

5 effluent concentrations
+ 1 control
= 6 concentrations
x 4 replicates
= 24 tanks
x 10 animals/replicate
= 240 animals

The test larvae should come from a pool of larvae consisting of at least three separate spawnings. To begin a test with five effluent concentrations and a control, each with four replicates, the minimum number of larvae needed is 240. You will need more than this to allow for extra larvae to choose from. The larvae are placed one or two at a time into the test chambers until each chamber contains ten larvae. To equalize the water volume added to each tank, the fish can be put in small beakers first. For example, place one or two at a time in a small beaker until five are in each. Then, reduce the water in each beaker to about 5 ml. Add these to each tank until ten fish are in each replicate.

Feeding

Artemia are available from
Aquarium Products,
180 L. Penrod Ct.,
Glen Burnie, MD 21061.

Once the test is set up, the animals are fed 0.1 ml of concentrated Artemia nauplii. The Artemia, or brine shrimp, should be started the day before testing begins. At 25°C, the brine shrimp will hatch in 16 to 18 hours. A fresh batch of brine shrimp should be prepared daily for the next day's use. Rinse the Artemia in freshwater and concentrate them in diluent water prior to each feeding. It is important that the larvae are fed 0.1 ml of the concentrate twice each day at least 6 hours apart to ensure live nauplii for the fish. Using less than 24 hour old Artemia ensures a small size and provides the highest nutritional value.

Ambient laboratory lighting is sufficient for fathead minnow testing, but it should be on a controlled regime of sixteen hours light and eight hours dark. Ambient laboratory conditions are acceptable if they meet minimum environmental control standards and there are no large scale fluctuations.

Renewal

A fathead survival count should be recorded daily and all dead larvae removed. One method used to facilitate counting and cleaning is a light box which illuminates the larvae. During this phase of the test, take care not to disturb the larvae too much. The easiest method to remove the day-old effluent is to start a small siphon and lower the test media to a depth of 7 to 10 ml while removing all food particulates. That leaves approximately 15 to 20% of the total volume. An opaque Tygon® Y-tube cut off at an angle works well as a syphon, and the dark color causes the fry to move away. Another method is to use a large pipette, 50 to 100 ml capacity, fitted with a rubber bulb.

Because of their small size, care must be taken not to remove any of the larvae. Collect the water as it is siphoned from the tanks, a white pan facilitates observing any fish that are inadvertently siphoned from the chambers during the cleaning operation. If a fish is siphoned out and is still in good condition, transfer it back to the test tank. If an animal is killed or injured, it should be duly noted and the animal removed. This changes the initial number of fish in that replicate.

To refill the tank pour the new test media slowly down the side of the test container. This will avoid excessive turbulence and prevent damage to the larvae.

Test Termination

The fish are not fed on day seven. A final survival count is made and the dead fish are removed. The remaining fish can either be weighed immediately or preserved in 70% alcohol for weighing later. It is extremely important that the fish be weighed within two weeks of test termination.

To determine the final weights, first the weigh boats are:

- o labelled;
- o dried; and
- o weighed for tare weight

The fish are rinsed with distilled water and all the fish from one replicate are placed in one container. Dry the fish at 100°C for at least 2 hours but less than 24. Weights should be obtained to the nearest 0.1 mg. After each group's weight is determined, it is divided by the actual number of fish weighed. If any fish are unaccounted for in the weighing process, the group weight should be divided by the number actually weighed--which could be different from the survival number. For the test to be acceptable, control survival must be at least 80% and the control mean weight at least 0.25 mg. The statistical analysis of the test results should be conducted according to the test manual.

Data Analysis

Data analysis procedures are presented in the appendices of the Chronic Methods Manual.

## OTHER PROCEDURAL CONSIDERATIONS

In addition to strict adherence to the test protocol, there are other important factors that may influence test results. Two of these are the choice of diluent water and the culturing of test animals. The diluent water that is used is an important consideration due to the fact that not all surface water is reliable water for culturing. Therefore, before initiating a test, it is important to establish the growth and survival rates are for each water source. For artificially reconstituted waters, it is very important to start with a "high purity" distilled and deionized water. This may mean installing a high grade filtering system and installing the filters in this order:

Diluent Water

- o ion exchange;
- o carbon filter;
- o organix-Q®; and
- o fine filter.

Also, avoid storing water for more than a month.

Methods for Measuring Acute Toxicity of Effluents to Freshwater and Marine Organisms

Good cultures are important for ensuring reliable test results. Culturing methods for fathead minnows are explained in the Acute Methods Manual and is the subject of a separate training tape.

# REFERENCES


- Short-term Methods for Estimating Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. USEPA Office of Research and Development. December 1985. EPA/600/4-85-014.

- Technical Support Document for Water Quality-based Toxics Control. USEPA Office of Water. September 1985. EPA/440/4-85-032.

- Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. USEPA Office of Research and Development. March 1985. EPA/660/4-85-013.

## GLOSSARY

**Acute toxicity.** An adverse effect measured in a short period of time (96 hours or less in toxicity tests). The effect can be measured in lethality or any variety of effects.

Artemia. Brine shrimp recommended as the food source. Brazilian or Columbian strains are preferred because the supplies are found to have low concentrations of chemical residues.

**Average mean dry weight.** All the fish exposed at one concentration are weighed together. The total dry weight is divided by the number of fish weighed to obtain the average mean dry weight.

**Chronic toxicity.** An adverse effect that occurs over a long exposure period. The effect can be lethality, impaired growth, reduced reproduction, etc.

**Diluent water.** Dilution water used to prepare the effluent concentrations.

**Effluent sample.** A representative collection of the discharge that is to be tested.

**Effluent concentrations.** Fathead minnows are exposed to different dilutions, or concentrations, of an effluent to determine the effects of the sample.

**Fathead minnow.** The species used is Pimephales promelas.

**Larvae.** The fathead minnow young are less than 24 hour old larvae at the start of the test.

**LC50.** The toxicant concentration killing 50% of the exposed organisms at a specific time of observation.

**Static renewal.** The daily replacement of effluent medium in the test chamber.

**Toxicity test.** A measure of the toxicity of a chemical or effluent using living organisms. The test measures the degree of response of an exposed organism to a specific chemical or effluent.

**Whole effluent toxicity.** The aggregate toxic effect of an effluent measured directly with a toxicity test.

## APPENDIX A

### APPARATUS AND EQUIPMENT LIST

Fathead minnow and brine shrimp culture units -- see the Acute Methods Manual. This test requires 150-300 newly hatched larvae. It is preferable to obtain these fish from an inhouse fathead minnow culture unit. If it is not feasible to culture fish inhouse, embryos or newly hatched larvae can be shipped in well oxygenated water in insulated containers.

Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-hour composite sample of 4 L.

Sample containers -- for sample shipment and storage.

Environmental chamber or equivalent facility with temperature control (25 ± 1°C).

Water purification system -- Millipore Super-Q or equivalent.

Balance -- analytical, capable of accurately weighing larvae to 0.0001 g.

Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

Test chambers -- borosilicate glass beakers or aquaria, or non-toxic disposable plastic labware. A minimum of three 1-L beakers or glass aquaria (7.6 cm wide x 16 cm long x 8.0 cm high) are required for each concentration and control. Aquaria can have a 7.4 x 7.0 cm piece of 60 mesh stainless steel or Nytex® screen glued 2.5 cm in across one end. The surface to volume ratios in 1-L beakers and the glass aquaria are approximately the same. To avoid potential contamination from the air, the chambers should be covered during the test.

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

Volumetric pipets -- Class A, 1-100 ml.

Serological pipets -- 1-10 ml, graduated.

Pipet bulbs and fillers -- Propipet®, or equivalent.

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

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

Glass or electronic thermometers -- for measuring water temperatures.

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

## APPENDIX A CONTINUED

National Bureau of Standards certified thermometer (see USEPA Method 170.1, USEPA 1979b).

pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

## APPENDIX B

### REAGENTS AND CONSUMABLE MATERIALS

Reagent water -- defined as activated-carbon-filtered distilled or deionized water that does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water.

Effluent, surface water, and dilution water.

Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1, USEPA 1979b).

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

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

Laboratory quality assurance samples and standards for the above methods.

Specific conductivity standards (see USEPA Method 120.1, USEPA 1979b).

Reference toxicant solutions.

Alcohol (4%) for uses as a preservative for the fish larvae.

Brine Shrimp (Artemia) Cysts -- see the Acute Methods Manual. Although there are many commercial sources of brine shrimp eggs, the Brazilian or Columbian strains are preferred because the supplies examined have had low concentrations of chemical residues. (One source is Aquarium Products, 180 L Penrod Ct., Glen Burnie, MD, 21061). Each new batch of Artemia cysts should be evaluated for nutritional suitability against known suitable reference cysts by performing a larval growth test. It is recommended that a sample of newly-hatched Artemia nauplii from each new batch of cysts be chemically analyzed to determine that the concentration of total organic chlorine does not exceed 0.15 ug/g wet weight or the total concentration of organochlorine pesticides plus PCBs does not exceed 0.3 ug/g wet weight. If those values are exceeded, the Artemia should not be used.

Limited quantities of reference Artemia cysts, information on commercial sources of good quality Artemia cysts, and procedures for determining cysts suitability are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, 45268.

Test organisms -- Newly-hatched fathead minnow larvae (See Acute Methods Manual).

## APPENDIX C

### RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST

| | | |
|---|---|---|
| 1. | Test type: | Static renewal |
| 2. | Temperature (°C): | 25 ± 1°C |
| 3. | Light quality: | Ambient laboratory illumination |
| 4. | Light intensity: | 10-20 uE/$m^2$/s (50-100 ft-c)(ambient lab levels) |
| 5. | Photoperiod: | 16 h light, 8 h darkness |
| 6. | Test chamber size: | 1-L beakers or glass aquaria |
| 7. | Test solution volume: | 250 ml/replicate |
| 8. | Renewal of test concentrations: | Daily |
| 9. | Age of test organisms: | Newly hatched larvae; <24 hours old |
| 10. | Larvae/test chamber and control: | 10 larvae/chamber; Minimum of 30 larvae/ test concentration |
| 11. | Replicate chambers/ concentration: | 4 (minimum of 3) |
| 12. | Feeding regime: | Feed 0.1 ml ≤ 24-hour old brine shrimp nauplii in a concentrated suspension twice daily, at least 6 hours between feedings |
| 13. | Cleaning: | Siphon daily, immediately before test solution renewal |
| 14. | Aeration: | None, unless DO concentration falls below 40% saturation. Rate should be less than 100 bubbles/min. |
| 15. | Dilution water: | Milli-Q or equivalent water is used to make a standard moderately hard water, or diluted mineral water (i.e 9 parts Milli-Q and 1 part Perrier® or equivalent). Aerate a minimum of 12 hours before use. |
| 16. | Effluent concentrations: | At least 5 effluent concentrations and a control |
| 17. | Dilution factor: | Approximately 0.3 or 0.5 |
| 18. | Test duration: | 7 days |

## APPENDIX C CONTINUED

| | | |
|---|---|---|
| 19. | Effects measured: | Survival and growth (increase in weight) |
| 20. | Test acceptability | 80% or greater survival in control solutions and a minimum weight of controls of at least .250 mg. |
| 21. | Sampling requirements: | Minimum of three 24-h composite samples. For on-site tests, sample should be used within 24 h of the time they are removed from the sampling device. For off-site tests, samples should be used within 36 h of collection. |
| 22. | Minimum sample volume required daily: | 2 L |