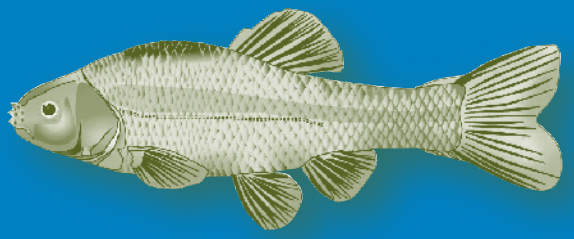
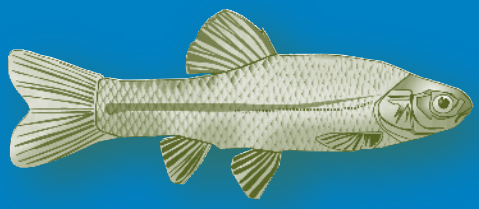
Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests

Supplement to Training Video





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Water Permits Division
1200 Pennsylvania Ave., NW
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EPA-833-C-06-001
December 2006

NOTICE

The revision of this report has been funded wholly or in part by the Environmental Protection Agency under Contract EP-C-05-046. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Foreword

This report serves as a supplement to the video "Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests" (EPA, 2006a). The methods illustrated in the video and described in this report support the methods published in the U.S. Environmental Protection Agency's (EPA's) Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition (2002a) and Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Fifth Edition (2002b), referred to as the Chronic and Acute Method Manuals, respectively. The video and this report provide details on initiating, renewing, and terminating tests based on the expertise of the personnel at the EPA's Mid-Continent Ecology Division (MED) in Duluth, Minnesota (EPA-Duluth).

This report and its accompanying video are part of a series of training videos produced by EPA's Office of Wastewater Management. The video entitled "Culturing of Fathead Minnows (*Pimephales promelas*)" (EPA, 2006b) complements the material in this video by explaining the method for culturing fathead minnows for use in toxicity tests. These videos are available through the National Service Center for Environmental Publications (NSCEP) at (800) 490-9198 or nscep@bps-lmit.com. Other available freshwater videos include "*Ceriodaphnia* Survival and Reproduction Toxicity Tests" (EPA, 2006c).

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Introduction

This report accompanies the Environmental Protection Agency's video training for conducting freshwater fathead minnow (*Pimephales promelas*) larval survival and growth toxicity tests (EPA, 2006a). The test method is found in Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (EPA, 2002a). The test is adapted from methods developed by Teresa Norberg-King and Dr. Donald Mount of EPA's Mid-Continent Ecology Division (MED), Duluth, Minnesota (Norberg and Mount, 1985). The material presented in both the video and this report summarizes the methods but does not replace a thorough review and understanding of the methods by laboratory personnel before conducting the test.

Background

Under the National Pollutant Discharge Elimination System (NPDES) program (Section 402 of the Clean Water Act), 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 (WET) 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 freshwater seven-day static renewal exposure for determining sublethal toxicity in order to estimate 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 larvae. Minnows that are 24 hours old or less are exposed, and growth is measured as the difference in the larvaes 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 Chronic Methods Manual.

Test Method

Effluent Sampling

Effluent sampling must be conducted according to the Chronic Methods Manual (EPA, 2002a) and any specific permit conditions. Samples are collected over a 24-hour period or when a 24-hour composite sampling period is completed. The time lapsed (holding time) from sample collection completion to first use of each grab or composite sample must not exceed 36 hours for test results to be acceptable for use in NPDES permit compliance testing. However, for all other testing purposes, no more than 72 hours should elapse between collection completion and first use of the sample. In static renewal tests, each grab or composite sample also may be used to prepare test solutions for renewal at 24, 48, and/or 72 hours after first use if stored at 0° – 6°C, with minimum head space. Also according to the 2002 promulgated methods, for WET samples with a specified storage temperature of 4°C, storage at a temperature above the freezing point of water to 6°C shall be acceptable (0° – 6°C). EPA has further clarified that hand-delivered samples used on the day of collection do not need to be cooled to 0° – 6°C prior to test initiation. (EPA, 2002c).

Section 8 of the Chronic Manual covers sample collection. Note that surface waters should be filtered (60 µm plankton net) for fathead minnow tests.

Dilution Preparation

To start a test, warm the effluent to 25° ± 2°C slowly to avoid exceeding the desired temperature. This is done using a water bath and monitoring the temperature closely. A temperature of 25° ± 1°C should be maintained throughout the 7-day test period and the instantaneous temperature must not deviate by more than 3°C during the test.

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 four replicates per concentration. The Chronic Methods Manual recommends the use of a 0.5 dilution factor, which provides precision of ± 100%. Test precision shows little improvement as the dilution factor is increased beyond 0.5, and declines rapidly if a smaller dilution factor is used.

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 (DO) at the beginning and end of each 24 hour renewal in at least one test chamber of each test concentration and in the control. If aeration is required, aerate all concentrations and the control. Take care not to cause excess turbulence that can cause physical stress to the organisms.

It is recommended that temperature be recorded continuously or observed and recorded in at least two locations in the environmental control system or the samples during the test.

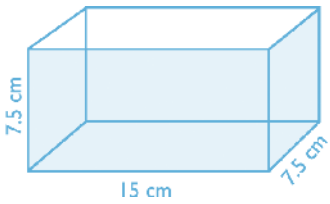
Dissolved oxygen, temperature, pH, and total residual chlorine must be measured on each new sample. EPA also recommends that total alkalinity, total hardness, and conductivity be measured on each new sample. Dissolved oxygen, temperature, and pH are measured at the beginning and end of each 24 hour renewal in at least one test chamber of each test concentration and in the control. Measuring conductivity at the beginning and end of each 24 hour renewal is preferred but not required. The temperature and pH of the effluent sample also must be measured each day before preparing the test solutions. See Table 1.

Table 1. Monitoring Schedule

Parameter	*Monitoring Frequency*		
	Each New Sample	*24 hour Exposure Period*	
		Beginning	*End*
Dissolved oxygen[1, 2]	X	X	X
Temperature[1, 2, 3]	X	X	X
pH[1, 2, 3]	X	X	X
Conductivity[1, 2]	X	X	X
Alkalinity[1]	X	X	
Hardness[1]	X	X	
Total Residual Chlorine[1]	X		

1 Measured in each new sample (100% effluent or receiving water) and in control.
2 Beginning and end measurement on one replicate in each concentration and the control.
3 Measured in the effluent sample each day before preparation of new test solutions.

Test Chambers



The Chronic Methods Manual recommends that test chambers should not be smaller than a 500 mL beaker, yet allows test chambers to be 1 L, 500 mL, or 250 mL plastic cups or fabricated rectangular (0.3 cm thick) glass chambers. The glass chambers should measure 15 cm by 7.5 cm by 7.5 cm high. 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. To avoid potential contamination from the air and excessive evaporation of test solutions during the test, the chambers should be covered with safety glass plates or sheet plastic (6 mm thick). Ambient laboratory lighting is sufficient for fathead minnow testing, but it should be on a controlled regime of 16 hours light and 8 hours dark. Ambient laboratory conditions are acceptable if they meet minimum environmental control standards and there are no large scale fluctuations.

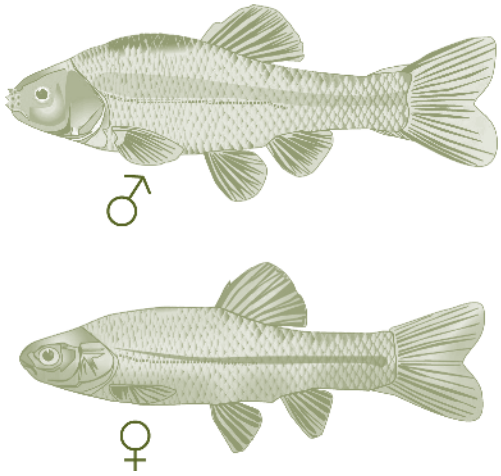


Figure 1. Male Fathead Minnow (top) and Female Fathead Minnow (bottom)

Test Organisms

The test larvae should come from a pool of larvae consisting of at least three separate spawnings (Figure 1). 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 240 to allow for extra larvae to choose from.

Calculation of Test Animals:
5 effluent concentrations
+1 control
= 6 concentrations
x 4 replicates
= 24 tanks
x 10 animals/replicate
= 240 animals

The larvae are placed one or two at a time into the test chambers until each chamber contains ten larvae. To minimize the water volume added to each tank, the fish can be put in small beakers first. For example, place one or two fish 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 fish to each tank until ten are in each replicate.

Feeding

Once the test is set up, the larvae are fed 0.1 g of concentrated *Artemia* nauplii three times per day or 0.15 g two times per day. 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 0.15 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.

Renewal

A fathead survival count must 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 mm 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 siphon, and the dark color causes the

larvae 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 in a white pan to facilitate observing any larvae that are inadvertently siphoned from the chambers during cleaning. If a larvae is siphoned out and is still in good condition, transfer it back to the test tank. If a larvae is killed or injured, it should be duly noted and the larvae 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 larvae 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 preserved larvae be weighed within two weeks of test termination. To determine the final weights, first the weigh boats are labeled; dried; and a tare weight measured. The fish are rinsed with distilled water and all the fish from one replicate are placed in one container. Dry the larvae at 100°C for at least 6 hours but less than 24 hours. Weights should be obtained to the nearest 0.01 mg. After each group's weight is determined, it is divided by the initial number of fish in that replicate. 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:
Complete data analysis procedures are presented in the appendices of the Chronic Methods Manual.

Test Acceptability and Data Review

Test data are reviewed to verify that test acceptability criteria (TAC) requirements for a valid test have been met. For instance, the TAC requires 80% or greater survival in controls with an average dry weight per surviving organism in control chambers equal to or exceeding 0.25 mg. However, the response used in the statistical analysis is mean weight per original organism for each replicate, which is a combined survival and growth endpoint that is termed "biomass." Any test not meeting the minimum TAC is considered invalid. All invalid tests must be repeated with a newly-collected sample. Further guidance is provided in the Chronic Methods Manual.

The test results must be reviewed for concentration-response relationships for all multi-concentration tests. The concentration-response relationship generated for each multi-concentration test must be reviewed to ensure that calculated test results are interpreted appropriately. In conjunction with this requirement, EPA has provided recommended guidance for concentration-response relationship review (EPA, 2000).

EPA's promulgated toxicity testing method manuals (2002 a, b) recommend the use of point estimation technique approaches for calculating endpoints for effluent toxicity tests under the NPDES program. The promulgated methods also require a data review of toxicity data and concentration-response data, and require calculating the percent minimum significant difference (PMSD) when point estimation (e.g., LC_{50}, IC_{25}) analyses are not used. EPA specifies the PMSD must be calculated when NPDES permits require sublethal hypothesis testing. EPA also requires that variability criteria be applied as a test review step when NPDES permits require sublethal hypothesis testing endpoints (i.e., no observed effect concentration [NOEC] or lowest observed effect concentration [LOEC]) and the effluent has been determined to have no toxicity at the permitted receiving water concentration (EPA, 2002b). This reduces the within-test variability and to increase statistical sensitivity when test endpoints are expressed using hypothesis testing rather than the preferred point estimation techniques.

Other Procedural Considerations

Diluent Water

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 testing and culturing. Therefore, before initiating a test, it is important to establish the growth and survival rates 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 the order of ion exchange, carbon filter, Organex-Q®, and fine filter. Also, avoid storing water for more than 14 days.

Test Organism Cultures

Good cultures are important for ensuring reliable test results. Culturing methods for fathead minnows are explained in the Acute Methods Manual (EPA, 2002b) and is the subject of a separate training video (EPA, 2006b).

References

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EPA, 2002a. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. Office of Water, Cincinnati, OH. EPA-821-R-02-013.

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EPA, 2002c. Final Rule. 40 CFR Part 136. Guidelines Establishing Test Procedures for the Analysis of Pollutants; Whole Effluent Toxicity Test Methods. 67 FR 69952–69972, November 19, 2002.

EPA, 2006a. Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Test, Training Video. EPA-833-C-06-001. December 2006.

EPA, 2006b. Culturing of Fathead Minnows (*Pimephales promelas*), Training Video. EPA-833-C-06-001. December 2006.

EPA, 2006c. *Ceriodaphnia* Survival and Reproduction Toxicity Tests, Training Video. EPA-833-C-06-001. December 2006.

Norberg, T.J. and D.I. Mount, 1985. A new fathead minnow (*Pimephales promelas*) subchronic toxicity test. Environ. Toxicol. Chem. 4(1): 711-718.

References are available online at **www.epa.gov/npdes**.

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.

Acute toxicity test. A test to determine the concentration of effluent or ambient waters that causes an adverse effect (usually death) on a group of test organisms during a short-term exposure (e.g., 24, 48, or 96 hours). Acute toxicity data are analyzed using statistical procedures (e.g., point estimate techniques or a t-test).

Artemia. The marine invertebrate (referred to as brine shrimp) used as the recommended 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 in a given test chamber (replicate) are weighed together. The total dry weight is divided by the original number of fish in the replicate 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 a NPDES permitted facility's discharge that is to be tested.

Effluent concentrations. Different dilutions, or concentrations, of an effluent used to determine the biological effects on test organisms (i.e., fathead minnows).

Fathead minnow. Freshwater vertebrate fish species (*Pimephales promelas*).

Larvae. Post-hatch fish that are not free-swimming and are morphologically immature (i.e., < 24 hour-old fathead minnows).

LC_{50} (lethal concentration, 50%). The concentration of toxicant or effluent that would cause death to 50% of the test organisms.

NPDES (National Pollutant Discharge Elimination System) Program. The national program for issuing, modifying, revoking, and reissuing, terminating, monitoring and enforcing permits, and imposing and enforcing pretreatment requirements, under Sections 307, 318, 402, and 405 of the Clean Water Act.

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

Toxicity test. A procedure to determine the toxicity of a chemical or effluent using living organisms. A toxicity test measures the degree of effect of a specific chemical or effluent on exposed test organisms.

WET (Whole Effluent Toxicity). The total 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 240–360 larvae. It is preferable to obtain larvae from an in-house fathead minnow culture unit. If it is not feasible to culture fish in-house, 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 5 L.

Sample containers. For sample shipment and storage.

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

Water purification system. Millipore® Milli-Q® deionized water, or equivalent.

Balance. Analytical, capable of accurately weighing larvae to 0.0000 1 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.

Borosilicate glass beakers or aquaria, or non-toxic disposable plastic labware. A minimum of four 500-mL beakers or glass aquaria (7.6 cm wide x 16 cm long x 8.0 cm high) are required for each concentration and 1 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 500 ml beakers and the glass aquaria are approximately the same. To avoid potential contamination from the air, the chambers should be covered during the test. The Methods Manual recommends that test chambers should not be smaller than a 500 mL beaker, yet allows test chambers to be 1 L, 500 mL, or 250 mL plastic cups or fabricated rectangular (0.3 cm thick) glass chambers. The glass chambers should measure 15 cm by 7.5 cm by 7.5 cm high.

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 inner diameter, for transferring larvae.

Wash bottles. For rinsing small glassware and instrument electrodes and probes.

Thermometers, glass or electronic, laboratory grade. For measuring water temperatures.

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

Thermometers. National Bureau of Standards Certified (EPA, 2002a), to calibrate laboratory thermometers.

Meters, pH, DO, and specific conductivity. For routine physical and chemical measurements.

Drying oven. 50° – 150°C range for drying larvae

Appendix B

Reagents And Consumable Materials

Reagent water. Defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

Effluent, surface water, and dilution water.

Reagents for hardness and alkalinity tests. (See EPA, 2002a).

pH buffers 4, 7, and 10. (Or as per instructions of instrument manufacturer) for standards and calibration check (see EPA, 2002a).

Membranes and filling solutions for DO probe. (See EPA, 2002a), or reagents, for modified Winkler analysis.

Laboratory quality assurance samples and standards. For calibration of the above methods.

Specific conductivity standards. (EPA, 2002a).

Reference toxicant solutions. Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCI), cadmium chloride (C_dCl_2), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$), are suitable for use in the NPDES Program and other Agency programs requiring aquatic toxicity tests.

Ethanol (70%) of formalin (4%). For use as a preservative for the fish larvae.

Brine Shrimp (*Artemia* sp.) Cysts. (EPA, 2002b.) 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. Each new batch of *Artemia* cysts must be evaluated for nutritional suitability against known suitable reference cysts by performing a side-by-side 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 μg/g wet weight or the total concentration of organochlorine pesticides plus PCBs does not exceed 0.30 μg/g wet weight. If those values are exceeded, the *Artemia* should not be used.

Test organisms. Newly-hatched fathead minnow larvae (EPA, 2002b).

Appendix C

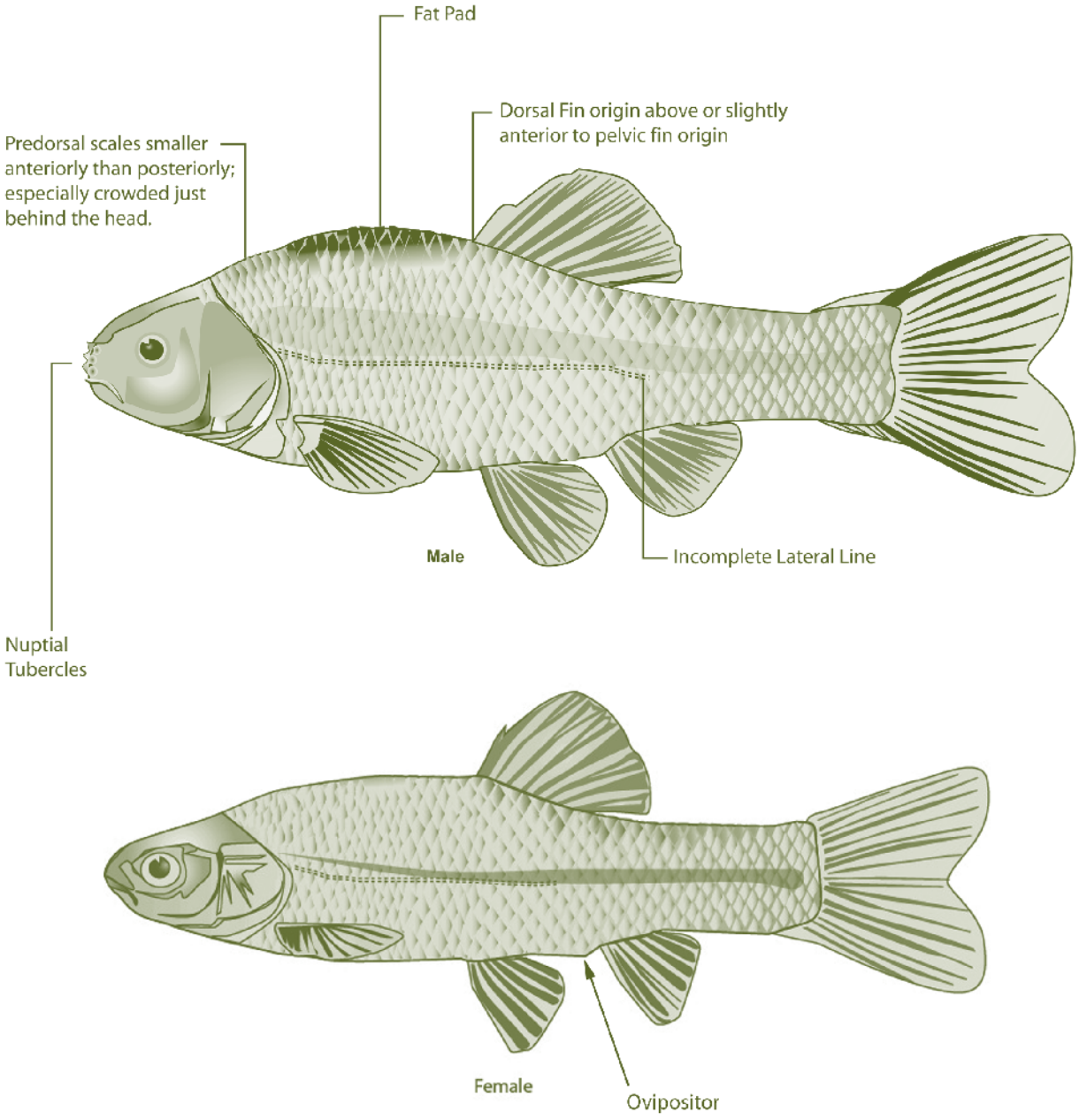
Summary of Test Conditions and Test Acceptability Criteria for Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests with Effluents and Receiving Waters (Test Method 1000.0)

Test type	Static renewal *(required)*
Temperature (°C)	25° ± 1°C *(recommended)* Must not deviate more than 3°C during the test *(required)*
Light quality	Ambient laboratory illumination *(recommended)*
Light intensity	10 – 20 uE/m^2/s (50 – 100 ft-c)(ambient lab levels) *(recommended)*
Photoperiod	16 hours light, 8 hours darkness *(recommended)*
Test chamber size	500 mL beakers or glass aquaria *(recommended minimum)*
Test solution volume	250 mL/replicate *(recommended minimum)*
Renewal of test concentrations	Daily *(required)*
Age of test organisms	Newly hatched larvae; < 24 hours old. If shipped, not more than 48 hours old; 24-hour range in age *(required)*
Larvae/test chamber and control	10 larvae/chamber *(required)*
Replicate chambers per concentration	4 *(required minimum)*
Feeding regime	On days 0 – 6, feed 0.1 g newly hatched (< 24-hour old) brine shrimp nauplii three times daily at 4-hour intervals, or as a minimum, 0.15 g twice daily at least 6-hour intervals (at the beginning of the work day and prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. *(recommended)*
Cleaning	Siphon daily, immediately before test solution renewal. *(required)*
Aeration	None, unless DO concentration falls below 4.0 mg/L. Rate not exceed 100 bubbles/minute. *(recommended)*
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. *(available options)*
Effluent concentrations	5 concentrations and a control *(recommended minimum)* Receiving water: 100% receiving water (or a minimum of 5) and a control *(recommended)*
Dilution factor	Effluents: ≤ 0.5 *(recommended)* Receiving waters: None or ≥ 0.5 *(recommended)*
Test duration	7 days *(required)*
Effects measured	Survival and growth (weight) *(required)*
Test acceptability	80% or greater survival in control; average dry weight per surviving organism in control chambers ≥ 0.25 mg. *(required)*
Sampling requirements	For on-site tests, samples collected daily, and used within 24 hours of the time they are removed from the sampling device. For off-site tests, a minimum of 3 samples (e.g., collected on day 1, 3 and 5) with a maximum holding time of 36 hours before first use. *(required)*
Sample volume required	2.5 L per day *(recommended)*

Source: EPA 2002a. Chronic Methods Manual. For the purposes of reviewing WET test data submitted under NPDES permits, each test conditions listed above is identified as required or recommended. See Subsection 10.2 of the Chronic Manual 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 these methods.

Appendix D:

Illustration of Fathead Minnow with Anatomical Identifications



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