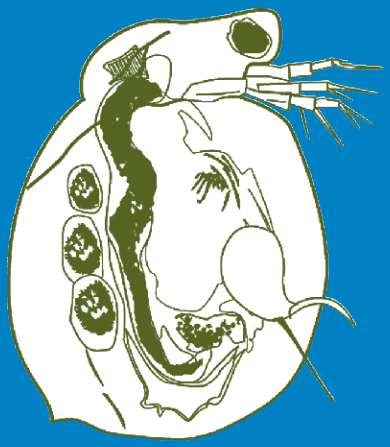
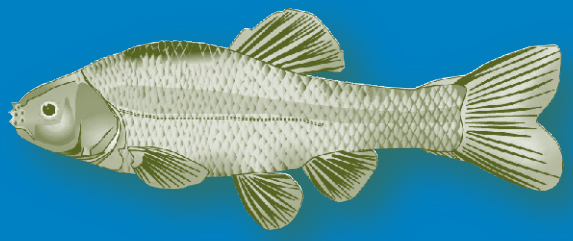
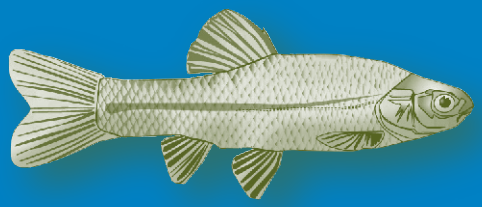
Whole Effluent Toxicity Training Video Series

Freshwater Series





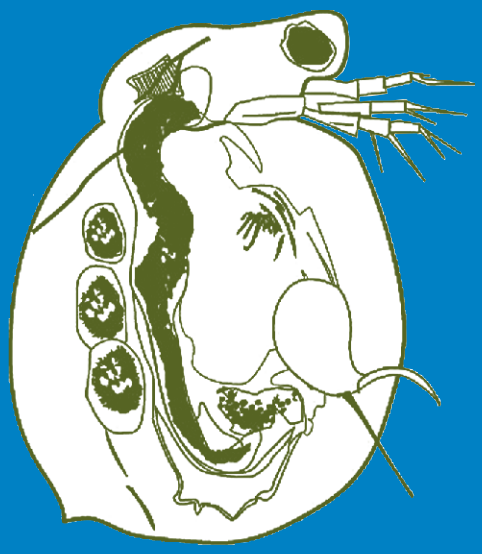


U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
1200 Pennsylvania Ave., NW
Washington, DC 20460

EPA-833-C-06-001
December 2006

Ceriodaphnia Survival and Reproduction Toxicity Tests

Supplement to Training Video



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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 "*Ceriodaphnia* Survival and Reproduction 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) referred to as the Chronic Methods Manual. 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 videos entitled "Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests" and "Culturing of Fathead Minnows (*Pimephales promelas*)" (EPA, 2006b,c) complement the material in this video by explaining the method for testing and culturing fathead minnows for use in freshwater whole effluent toxicity tests. These videos are available through the National Service Center for Environmental Publications (NSCEP) at (800) 490-9198 or **nscep@bps-lmit.com**.

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FIGURE

Introduction

This report accompanies the Environmental Protection Agency's video training for conducting *Ceriodaphnia dubia* (freshwater water flea) survival and reproduction 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*, Fourth Edition (EPA, 2002a). The test is adapted from methods developed by Dr. Donald Mount and Teresa Norberg-King of EPA's Mid-Continent Ecology Division (MED), in Duluth, Minnesota. 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 control pollutant discharges on a whole effluent toxicity (WET) basis, rather than by a chemical specific approach.

The test method requires a static renewal exposure. Every 24 hours, the Ceriodaphnia are transferred to a new test chamber containing a freshly prepared solution of the appropriate effluent concentration.

The test method requires a static renewal exposure system (Appendix A). Every 24 hours the *Ceriodaphnia dubia* are transferred to a new test chamber containing a freshly prepared solution of the appropriate effluent concentration. This report covers the general procedures used for conducting *Ceriodaphnia* toxicity tests. The *Ceriodaphnia* short-term chronic test estimates the toxicity of an effluent by exposing test organisms to different concentrations of effluent. The test results are based on survival and reproduction of the organisms using static renewal system. This report and the accompanying video describe how the test is set up, initiated, monitored, renewed, and terminated, followed by suggestions on maintaining healthy test organisms.

Test Method

Culturing

The first step is to set up *Ceriodaphnia* cultures that produce the young, called neonates, for the test. Mass culturing can be used to maintain large laboratory stocks, but individual brood cultures must be started at least three weeks before brood animals are needed for testing. The survival records of these individual brood cultures must be maintained to assure that healthy animals are used for testing. Brood animals are fed daily and the culture media is renewed at least three times a week.

Cultures produce Brood animals that produce Test animals.

For a test with 5 concentrations of effluent and a control, with 10 replicates per concentration, 10 brood cups with 8 or more young of the right age are randomly selected from a brood board. Test organisms must be less than 24 hours old and within 8 hours of the same age. Using neonates produced within 2 or 4 hours of each other is even more desirable. In order to obtain a sufficient number of test animals within this age specification, it is advisable to have at least 50 to 60 brood animals to select from. Neonates must be taken only from adults in individual cultures that have eight or

Five effluent concentrations
+1 control
= 6 concentrations
X 10 replicates
= 60 test vessels
X 1 brood animal/replicate
= 60 brood animals

more young in their third or subsequent brood. Adults in these individual brood cultures can be used as brood stock until they are 14 days old.

Effluent Sampling

Effluent sampling should be conducted according to the Chronic Methods Manual (EPA, 2002a) and any conditions specified in a regulatory permit. Samples are collected over a 24 hour period or when a 24 hour composite sampling period is completed. If the data from the samples are to be acceptable for use in the NPDES program, the lapsed time (holding time) from sample collection to first use of each grab or composite sample must not exceed 36 hours. However, in no case should more than 72 hours lapse between collection and first use of sample. In the USEPA Federal register notice (2002b), EPA clarified the allowable sample holding temperatures for WET samples as 0 – 6°C and 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.

Samples must be collected as described in the methods manual or as required by a permitting authority. Samples cannot be older than 72 hours for first use. Samples may be used for renewal at 24, 48, and/or 72 hours after first use.

Dilution Preparation

Warm the effluent to 25 ± 1°C slowly to avoid exceeding the desired temperature. Maintain this temperature throughout the test. Once the effluent and the dilution water have reached the desired temperature, the dilutions can be prepared. EPA recommends the use of five exposure concentrations and a control for each test using a 0.5 dilution series, with 10 replicates per concentration, 15 ml of medium and one organism per replicate. Sufficient test solution, for example 500 mL, should be prepared at each concentration to provide additional volume for chemical analyses of each test concentration and the control.

Test Organisms

On the day of the test, neonates for testing are obtained by removing any young from the culture beakers or transferring the brood animals to new beakers early in the morning on the day the test begins. Separate the brood cups with at least 8 young per female by 2- to 4-hours blocks, until at least 10 brood cups have sufficient young. If the neonates are held more than 1- to 2-hours before being used in the test, they should be fed appropriate amounts of the yeast, cereal leaves and trout food (YCT) and algal concentrate. (See feeding section below.) Be sure to record the age range of test organisms, the source of the neonates, and any feeding that occurs on test data sheets. If a brood animal is not producing three broods in seven days with 8 to 14 young in the brood, it may be overcrowded, underfed, or stressed in some way.

The recommended method for conducting the test is to use a randomized block design of treatment. This is done by randomly selecting 10 brood females with more than 8 young each. The neonates from each female are then assigned to one replicate of each effluent concentration. This way, each replicate of all of the concentrations is conducted with a neonate from a different brood animal. This procedure allows for tracking the performance of the young from each female (Figure 1). Also, if a female produces one weak offspring, or male, the likelihood of producing all weak or all male offspring is greater. Therefore, by using this known parentage technique, poor animal performance can be omitted from all concentrations for one female's young, thus decreasing variability among replicates. When using the randomized block designed, test chambers are randomized once at the beginning of the test.

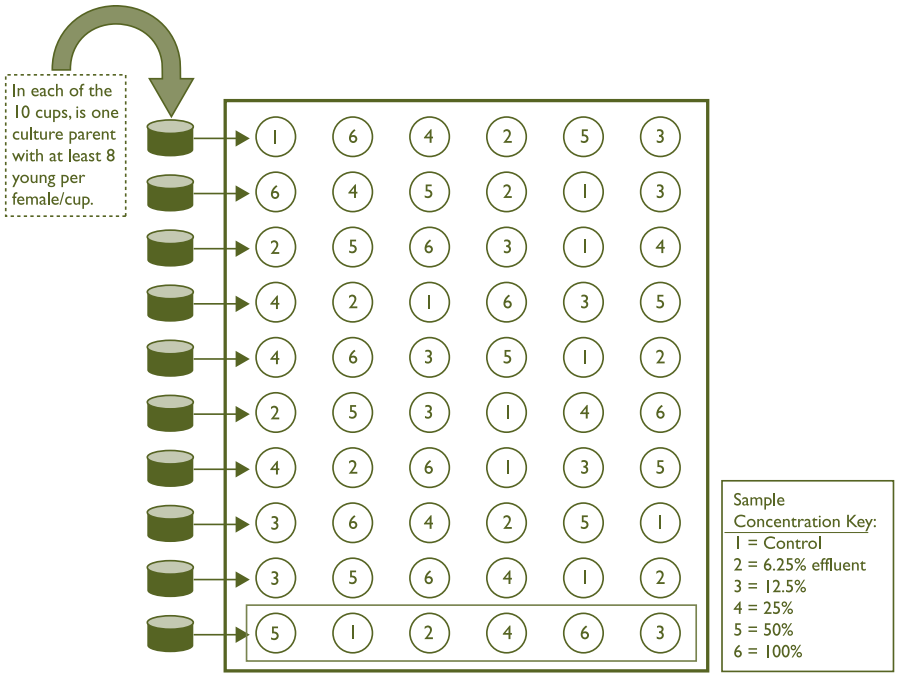

Figure 1. Test board as randomized for test conecentrations and adding young to begin test.

Routine Chemistries

Once the test concentrations are prepared, set aside an aliquot of each for the routine chemistries that must be performed. This allows for the chemistries to be performed without contaminating the actual test solutions with the probes. For test initiation and renewals, measure and record the dissolved oxygen (DO) at the beginning of each 24 hour renewal in each test concentration. This ensures that the dilutions are not supersaturated or below 4.0 mg/L. If they are, they should be aerated gently for a short period of time. DO measurements also should be performed at the end of each 24 hour exposure period for one replicate in each concentration and the control.

EPA recommends that the test temperatures be recorded continuously during the test. Temperature should be monitored continuously or observed and recorded daily for at least two locations in the environmental control system or the samples.

DO, 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 (Appendix C). DO, temperature, and pH are measured at the beginning and end of each 24 hour renewal in at least one test chamber for 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 (Table 1).

Feeding

The test animals are fed daily with the same food that the cultures receive. At test initiation and at the time of the daily effluent renewal, 0.1 mL of the YCT food mixture and 0.1 mL of algae are added to each 15 mL of test medium. The YCT food mixture is made of yeast, cereal leaves, and trout food (Appendix D). The algae is *Pseudokirchneriella subcapitata* (former species name was *Selenastrum capricornutum*). In the 15 mL test volume, the number of algae cells for feeding should be approximately 2 to 2.3 x 10^5 cells per mL.

See Other Procedural Considerations and Appendix D for Food Preparation Instructions

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		
Ammonia[4]	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.
4 If toxicity may be contributed by total ammonia (> 5mg/l), ammonia is measured on 100% effluent.

Between renewals, cover the test cups to prevent evaporation, as well as possible contamination from the laboratory environment. Glass is recommended because plexiglass can warp with moisture. Place the test trays in a temperature and photoperiod-controlled room or chamber and conduct the test at a temperature of 25° ± 1°C. Maintaining the temperature is important due to possible effects on reproduction. Control the photoperiod of 16 hours light and 8 hours dark. Also, light intensity and quality should be at a minimum of standard ambient laboratory conditions.

Renewal

Once the test is initiated, the renewals on days two through six are performed in the same manner as the test start. To do the daily renewals, prepare the test solutions, distribute the solutions to clean test containers using the **same** randomization layout used at test initiation and add food to each container. Test animals are then transferred with a glass dropper or pipette, releasing the animal under the surface of the water. As young may be present on day three, exercise care to transfer only the adult and not the young and do not dispose of the test cups until the young have been counted. If a test animal is injured or killed during the renewal process, it should be discarded and recorded. By killing one animal, you have reduced the initial number of test organisms for that treatment by one.

Record observations daily on *Ceriodaphnia* survival and reproduction. Three or four broods are usually obtained from the control animals during the six to eight-day test period, provided that the control water is reliable water. The first brood of 3 to 5 young is usually released on day three or four, with successive broods released every 30 to 36 hours thereafter. When counting the young, organisms are observed more readily if viewed against a black background or by using a low power dissecting microscope. When using a microscope, a 15 ml water volume in the beaker or plastic cup allows viewing the entire water column without adjustments to the microscope. Using a bottom light source also helps.

Test Termination

In the absence of toxicants, adults typically will produce an average of 15 to 35 young in the first three broods. Some effluents or toxicants may cause the young to be aborted. Tests should be terminated when 60% or more of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first. Because of the rapid rate of development of *Ceriodaphnia*, complete all observations on organism survival and numbers of offspring within 2 hours of test termination.

For the test to be considered acceptable, the control animals must have a survival rate of at least 80% or better, produce an average of 15 young per surviving female, and 60% of surviving control females must have produced three broods. In this three-brood test, offspring from fourth or higher broods should not be counted or included in test results. Statistical analyses of the test results should be conducted according to the Chronic Methods Manual (EPA, 2002a).

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 60% of surviving organisms producing 3 broods of at least 15 total neonates. 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 (EPA, 2002a).

Data analysis procedures are presented in the appendices of the Chronic Methods Manual (EPA, 2002a)

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, 2000a).

EPA's promulgated toxicity testing method manual recommends 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. To reduce the within-test variability and to increase statistical sensitivity when test endpoints are expressed using hypothesis testing rather than the preferred point estimation techniques, 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, 2002a).

Other Procedural Considerations

Food Preparation

In addition to strict adherence to the test protocol, there are other factors that may influence test results. The first is food preparation (see Appendix E – Food Preparation). It is important that the YCT mixture is similar in composition from week to week. To prepare the food, follow the general guideline in the Chronic Methods Manual, which is briefly described here:

Trout Food is prepared first. Five grams of trout food starter granules or ⅛" pellets are added to 1 liter of MILLI-Q® or equivalent water and mixed well in a blender. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Once the trout food has been digested, place in a refrigerator and allow it to settle for a minimum of 1 hour. The supernatant is then filtered through a Nytex® 110 mesh to remove the larger particulates. Aliquots of this food are used fresh or stored in the freezer for later use.

Cereal leaves of the YCT are prepared 24 hours in advance. Put 5.0 g of cereal leaves in 1 liter of the same diluent water, e.g., MILLI-Q®, as was used for the trout chow fermentation. Mix in a blender at high speed for 5 minutes, or stir on a stir plate for 24 hours at a moderate rate. If a

blender is used to suspend the material, place in a refrigerator overnight to settle. If a stir plate is used, allow to settle for 1 hour. Decant and filter the cereal leaves through a Nytex® 110 mesh. Cereal leaves are a powder of dehydrated cereal or grass leaves with Vitamin A, B2, C, and K.

Yeast for the YCT is mixed in the same diluent water as the other food items. Five grams of yeast should be placed in 1 liter of the MILLI-Q® water and mixed with a blender at low speed, shaked vigorously by hand, or stirred with a magnetic stirrer until the yeast is well dispersed. The yeast should be made up on the same day that all of the components are put together and added while in suspension.

After all three components are ready, mix equal volumes together and shake well. Measuring and adjusting suspended solids in the YCT (1.7 – 1.9 g solids/L) ensure that the food level is similar from batch to batch. Freshly prepared food can be used immediately (a maximum of two weeks if stored in the refrigerator between feedings), or it can be frozen until needed (not more than 3 months).

Algae is fed as a supplement to the YCT. For culturing the *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), the media from the Chronic Methods Manual is recommended. Again, the cell density of the algae must be monitored with each batch by using an electronic particle counter, a microscope and hemacytometer, fluormeter, or spectrophotometer. The algae cells should be monitored occasionally to be sure that the culture is not contaminated with any other types of algae. Algal concentrate may be stored in the refrigerator for two weeks.

Culturing Techniques

Culturing the test organisms is an important factor for accurate testing. To provide cultures of overlapping ages, new cultures are started weekly, using neonates from adults who produce at least eight young in their third or fourth brood. The adults can be used as sources of neonates until 14 days of age. A minimum of two ages of cultures are maintained concurrently to provide backup supplies of organisms in case of problems. Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less). Typically, 60 adult females will produce more than the minimum number of neonates (120) required for two tests. Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults or less than an average of 20 young per female would indicate problems, such as poor quality of culture media or food. Cultures that do not meet these criteria should not be used as a source of test organisms.

Low production during the three-brood test may be due to the health of the cultures, or factors such as the techniques of the lab personnel. One factor may be unfamiliarity of laboratory personnel with culturing techniques. Another may be that the brood animals were not adequate for producing test animals. To avoid these problems, a great deal of effort should be put into culturing. EPA recommends that laboratories maintain records on the brood animals to monitor their health. Count young production for seven days each month for 10 animals in the culture to determine how well the culture is performing.

It is also important that cultures are fed daily and that cultures are routinely changed (e.g., on Monday, Wednesday, and Friday) or the animals will quickly become overcrowded with their own young. A general guideline is to put as much effort into culturing as into testing because the success of the test relies on good culturing techniques.

Keep in mind that mass cultures are used only as a "backup" reservoir of organisms. If these animals are used as the source of brood organisms for individual culture, they should be maintained in good condition by frequent renewal with new culture medium at least twice a week for two weeks.

EPA recommends that chronic toxicity tests be performed monthly with a reference toxicant. *Ceriodaphnia* neonates, less than 24 hours old and all within 8 hours of the same age, are used to monitor the chronic toxicity of the reference toxicant to the *Ceriodaphnia* produced by the culture unit (see the Chronic Methods Manual for specific requirements).

Records that should be maintained are:

- source of organisms used to start the cultures
- type of food and feeding times
- dates culture were thinned and restarted
- rate of reproduction in individual cultures
- daily observations of the condition and behavior of the organisms in the cultures, and
- dates and results of reference toxicant tests performed.

Testing laboratory must perform at least one acceptable reference toxicant test per month for each type of toxicity test method conducted in that month.

Diluent Water

Another factor that is important to the success of the test is the choice of water to use as the diluent. Not all surface water is reliable for culturing. It is important to establish what the young production and survival rates are for each water that is used, before initiating a test. Maintaining survival records on the brood animals is helpful. To determine how well a culture performs, count young production for seven days each month for 10 animals in the culture. For artificially reconstituted waters, it is very important to start with a "high purity" distilled, deionzed or carbon filtered water. To achieve this install a high grade filtering system and install the filters in the following order:

- ion exchange
- ion exchange
- carbon filter
- Organex-Q®

Follow these with a final bacteria filter. Also, do not store water for more than 14 days.

Finally, if an adult does not produce young during the test, it should be put on a slide and its sex determined. This is relatively easy and is to be performed according to the guidelines in the Chronic Methods Manual.

Additional Requirements

Test review is an important part of an overall quality assurance program and is necessary for ensuring that all test results are reported accurately. Test review should be conducted on each test by both the testing laboratory and the regulatory authority. To do this, the collection and handling of samples are reviewed to verify that the sampling and handling procedures were followed. For WET test data submitted under NPDES permits, all required test conditions must be met or the test is considered invalid and must be repeated with a newly collected sample. The Chronic Methods Manual (EPA, 2002a) provides some guidance on how to handle deviations from recommended test conditions that must be evaluated on a case-by-case basis to determine the validity of test results. Chain-of-custody forms are reviewed to verify that samples were tested within allowable sample holding times. Any deviations from the procedures are to be documented and described in the data report. Next, the test data are reviewed to verify that

TAC requirements for a valid test have been met. Any test not meeting the minimum test acceptability criteria is considered invalid. All invalid tests must be repeated with a newly collected sample. The test conditions are reviewed and compared to the specifications listed in the summary of test condition tables (Appendix A). Physical and chemical measurements taken during the test (e.g., temperature, pH, and DO) are reviewed and compared to specified ranges. Any deviations from TAC specifications should be documented and described in the data report. The statistical methods used for analyzing test data are reviewed to verify that the recommended flowcharts for statistical analysis were followed. Any deviation from the recommended flowcharts for selection of statistical methods should be noted in the data report.

EPA recommends control charts be prepared for each combination of reference toxicant, test species, test conditions, endpoints, and for the PMSDs calculated for successive effluent tests (EPA, 2000). The TAC, test conditions, concentration-response relationship, and test sensitivity of the reference toxicant test are reviewed to verify that the reference toxicant test conducted was valid. Toxicity endpoints from five or six tests are adequate for establishing the control charts. Laboratories should compare the calculated coefficient of variation, CV (i.e., standard deviation/mean) of the IC_{25} for the 20 most recent data points to the distribution of laboratory CVs reported nationally for reference toxicant testing.

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

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

Glossary

Acute toxicity. An adverse effect measured on a group of test organisms during a short-term exposure 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.

Algae. The algae used is *Pseudokirchneriella subcapitata* (former species name *Selenastrum capricornutum*).

Cereal Leaves. A powder of dehydrated cereal or grass leaves used in the food mixture.

***Ceriodaphnia*.** The freshwater invertebrate cladoceran, *Ceriodaphnia dubia* (water flea), used for this test method.

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

Coefficient of Variation (CV) is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean. Coefficient of variation is a measure of precision within (intralaboratory) and among (interlaboratory) laboratories.

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

Effluent concentrations. Concentrations or dilutions of an effluent sample to which *Ceriodaphnia* are exposed to determine the biological effects of the sample on the test organism.

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

Hypothesis Testing. Technique (e.g., Dunnett's test) that determines what concentration is statistically different from the control. Endpoints determined from hypothesis testing are NOEC and LOEC.

Inhibition Concentration (IC). IC is a point estimate of the toxicant concentration that would cause a given percent reduction (i.e., IC_{25}) in a non-quantal biological measurement (e.g., reproduction or growth) calculated from a continuous model.

Lethal concentration (i.e. LC_{50}). The toxicant concentration resulting in a reduction of survival equal to 50% of the exposed organisms at a specific time of observation.

Lowest Observed Effect Concentration (LOEC). The LOEC is the lowest concentration of toxicant to which organisms are exposed in a test, which causes statistically significant adverse effects on the test organisms (i.e., where the values for the observed endpoints are statistically significantly different from the control). The definitions of NOEC and LOEC assume a strict dose-response relationship between toxicant concentration and organism response.

Minimum Significant Difference (MSD). The MSD is the magnitude of difference from the control where the null hypothesis is rejected in a statistical test comparing a treatment with a control. MSD is based on the number of replicates, control performance and power of the test. MSD is often measured as a percent and referred to as PMSD.

No Observed Effect Concentration (NOEC). The NOEC is the highest tested concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle (short-term) test, that causes no observable adverse effect on the test organism (i.e., the highest concentration of toxicant at which the values for the observed responses are not statistically significantly different from the controls). NOECs calculated by hypothesis testing are dependent upon the concentrations selected.

Point Estimation Techniques. This technique is used to determine the effluent concentration at which adverse effects (e.g., fertilization, growth or survival) occurred, such as Probit, Interpolation Method, Spearman-Karber. For example, a concentration at which a 25% reduction in reproduction and survival occurred.

Neonate. Newly released *Ceriodaphnia* (less than 24 hours old) used for testing.

Receiving Water Concentration (RWC). The RWC is the concentration of a toxicant or the parameter toxicity in the receiving water (i.e., riverine, lake, reservoir, estuary or ocean) after mixing.

Static renewal. The exposure medium is replaced each day by moving the test animal to a new test cup prepared with the proper effluent concentration.

Toxicity test. A test to measure 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.

Trout food. The trout food can be flakes or pellets, and should follow the USFWS Specifications.

Whole effluent toxicity (WET). The aggregate toxic effect of an effluent measured directly with a toxicity test.

Yeast. The yeast used for feeding is common dry yeast.

Appendix A

Summary of Test Conditions and Test Acceptability Criteria

Test type	Static renewal *(required)*
Temperature (°C)	25° ± 1°C recommended test temperature should not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test *(required)*
Light quality	Ambient laboratory illumination *(recommended)*
Light intensity	10 – 20 uE/m^2/s, or 50 – 100 ft-c (ambient laboratory levels) *(recommended)*
Photoperiod	16 hours light, 8 hours darkness *(recommended)*
Test vessel size	30 mL *(recommended minimum)*
Test solution volume	15 mL *(recommended minimum)*
Renewal of test concentrations	Daily *(required)*
Age of test organisms	Less than 24 hours; and all released within a 8-hour period *(required)*
No. neonates per test chamber	1 assigned using blockings by known parentage *(required)*
No. replicate test chambers per concentration	10 *(required minimum)*
No. neonates per test concentration	*10 (required minimum)*
Feeding regime	Feed 0.1 ml each of the YCT and algal suspension per test chamber daily *(recommended)*
Cleaning	Use freshly cleaned glass beaker or new plastic cups daily *(recommended)*
Aeration	None *(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 dilute mineral water *(available options)*
Test Solutions	Effluents: 5 and a control *(required 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	Until 60% or more of surviving control females have three broods (maximum test duration 8 days) *(required)*
Effects measured	Survival and reproduction *(required)*
Test acceptability	80% or greater survival of control organisms and an average of ≥ 15 or more young per surviving female in the control solutions. 60% of surviving control females must produced three broods *(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 three 24 hour composite samples with a maximum holding time of 36 hours before first use. *(required)*
Minimum sample volume daily	1 L/day *(recommended)*

Appendix B

Reagents And Consumable Materials

Algae – Starter cultures of the green algae are available from commercial suppliers.

Data Sheets (one set per test) – for recording the data.

Effluent, surface water, and dilution water – Dilution water that contains undesirable organisms, that may attack the test organisms should be filtered through a fine mesh net (60-um or smaller openings).

Laboratory quality assurance samples and standards for the above methods.

Markers, waterproof – for marking containers.

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

pH buffers – Standard buffers at pH's of 4, 7, and 10 (or as per instructions or instrument manufacturer) for standards and calibration check (see EPA, 2002a).

Reagent water – defined as 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.

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

Reference toxicant solutions. Reference toxicants such as sodium chloride (NaCl), potassium chloride (KCI), cadmium chloride ($CdCl_2$), 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.

Sample Containers – for sample shipment and storage.

Specific conductivity standards (see EPA, 2002a).

Tape, colored – for labeling test vessels.

Vials, marked – for preserving specimens for verification.

Yeast, cereal, and trout food (YCT) or equivalent substitute food – for feeding the cultures and test organisms.

Appendix C

Apparatus And Equipment List

Balance – Analytical, capable of accurately weighing 0.00001 g.

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

Counting Method for Algae – Electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometerto measure cell density (cells/mL) in the algal food concentrate.

Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, ≥ 2-mm inner diameter, for transferring organisms.

Dissecting microscope – for examining and counting the neonates in the test chambers.

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

Filtering apparatus – for membrane and/or glass fiber filters.

Glass or electronic thermometers – for measuring water temperatures.

Light box – for illuminating organisms during examination.

Light meter – with a range of 0 – 200 μE/m2/s (0-1000 ft-c).

Mechanical shaker or magnetic stir plates

National Bureau of Standards Certified thermometer (see EPA, 2002a).

pH, DO, and specific conductivity meters – for routine physical and chemical measurements.

Pipets bulbs and fillers – Propipet®, or equivalent.

Pipettors, adjustable volume repeating dispensers – for feeding. Pipettors such as the Gibson REPETMAN®, Eppendorf®, Oxford®, or equivalent, provide a rapid and accurate means of dispensing small volumes (0.1 mL) of food to large numbers of test chambers.

Racks for test vessels – Racks approximately 8 cm x 40 cm, drilled to hold 10 test vessels for each concentration.

Randomization Templates – Used for randomizing the placement of test concentrations for blocked randomization. Templates are prepared from poster paper and covered in plastic, made by laboratory

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

Sample containers – for sample shipment and storage.

Samplers – Automatic sampler, preferably with sample cooling capability, capable of collecting a 24 hour composite sample of 5 L or more.

Serological pipets – 1 – 10 mL, graduated.

Test Chambers – 10 test chambers are required for each concentration and control. Test chambers such as 30-ml borosilicate glass beakers or disposable polystyrene cups are recommended because they will fit in the viewing field of most stereoscopic microscopes.

Volumetric flasks and graduated cylinders – Class A, borosilicate glass or non-toxic plastic labware, 10 – 1000 mL, for culture work and preparation of test solutions.

Volumetric pipets – Class A, 1 – 100 mL.

Wash bottles – for rinsing small glassware and instrument electrodes and probes.

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

Appendix D

Food And Algae Preparation

Feeding the proper amount of the right food is extremely important in *Ceriodaphnia* culturing and testing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food. Excess food could clog the filtering apparatus of the organisms or greatly decrease the DO and subsequently cause decreased young production and possibly death of the animals. A suspension of yeast, cereal leaves, and trout food (YCT) along with the algae will provide adequate nutrition when fed daily.

The YCT is prepared from three ingredients as follows:

Digested trout food or substitute flake food (Tetramin®, Bioril®, or equivalent), is prepared as follows:

1. Preparation of trout food or substitute flake food requires one week. Use starter or No. 1 pellets prepared according to current U.S. Fish and Wildlife specifications (EPA, 2002a)

2. Add 5.0 g trout food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 hour. Filter the supernatant through a fine mesh screen (i.e., Nytex® 110 mesh). Combine with equal volumes of supernatant from cereal leaves and yeast preparations (below). The supernatant can be used fresh or frozen until use. Discard the sediment.

Cereal leaves (powdered or dried cereal leaves) are prepared as follows:

1. Place 5.0 g of dried, powdered, cereal or alfalfa leaves, or rabbit pellets, in a blender. Cereal leaves or equivalent are available from commercial stores. Dried, powdered, or alfalfa leaves may be obtained from health food stores, and rabbit pellets are available at pet stores.

2. Add 1-L of MILLI-Q® water or equivalent.

3. Mix at high speed for five minutes in a blender or stir with a magnetic stirrer at medium overnight.

4. If blended, store in refrigerator overnight. If a magnetic stirrer is used, allow to settle for 1 hour.

5. Decant the supernatant through a Nytex® 110 mesh screen and combine with equal volumes of supernatant from the trout food (above) and yeast preparations (below). Discard any excess.

Yeast is prepared as follows:

1. Add 5.0 g of dry yeast to 1-L of MILLI-Q® water or equivalent.

2. Stir with a magnetic stirrer, shake vigorously by hand or use a blender at low speed until the yeast is well dispersed.

3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout food and cereal leaf preparations (above). Discard the remainder.

Combined foods as follows for the preparation of YCT:

1. Mix equal (300 mL) volumes of the three foods.
2. Measure suspended solids on each batch of YCT before feeding. Acceptable solids levels are between 1700 and 1900 mg/L. Record the date the food is prepared and the suspended solids concentration.
3. Place aliquots of the final mixture in small (50 ml to 100 mL) screw-cap plastic bottles and freeze until needed, but no more than three months.
4. Fresh or thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.

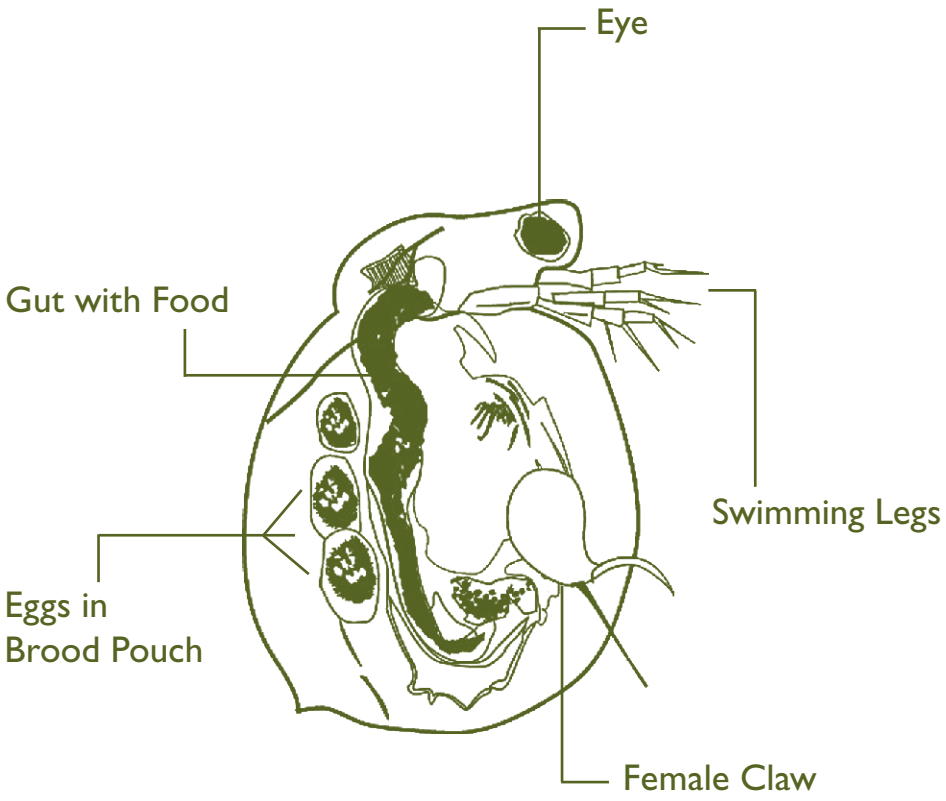
The algal concentrate is prepared as follows:

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for at least three weeks and siphoning off the supernatant (See Chronic Methods Manual for description of food culture preparation).
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 X 10^7/mL.
3. Assuming a cell density of approximately 1.5 X 106 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7 – 10 day culture will provide 4.5 X 109 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia dubia* tests.
4. Algal concentrate may be stored in the refrigerator for two weeks.

The quality of each batch of food prepared with a new supply of components should be determined by using the food in a 7-day reproduction test with control water.

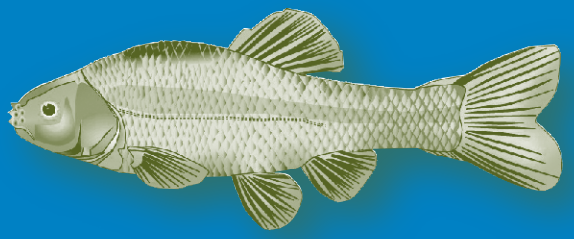
Appendix E

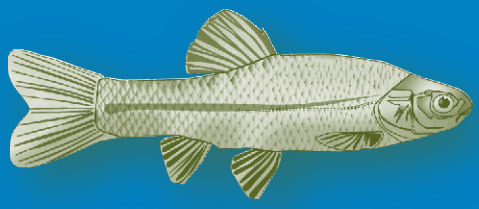
Ceriodaphnia dubia Anatomy



Culturing of Fathead Minnows (*Pimephales promelas*)

Supplement to Training Video





U.S. Environmental Protection Agency
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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 "Culturing of Fathead Minnows (*Pimephales promelas*)" (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) Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Fifth Edition (2002a) and Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Edition (2002b), referred to as the Acute and Chronic Methods Manuals, respectively. The video and this report provide details on setting up and maintaining cultures based on the expertise of the personnel at the EPA's Mid-Continent Ecology Division (MED) in Duluth, Minnesota (EPA-Duluth). More information can also be found in Guidelines for the Culture of Fathead Minnows (*Pimephales promelas*) for Use in Toxicity Tests (EPA, 1987).

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 "Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Test" (EPA, 2006b) complements the material in this video by explaining the 7-day subchronic toxicity test method. These videos are available through the National Service Center for Environmental Publications (NSCEP) at (800) 490-9198 or **nscep@bps-lmit.com**. Other freshwater videos include "*Ceriodaphnia* Survival and Reproduction Toxicity Tests" (EPA, 2006c).

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FIGURES

Introduction

Fathead minnows (*Pimephales promelas*) have been cultured for use in aquatic toxicity tests for over 30 years, and are the most common fish species used to determine sublethal toxicity of chemicals and complex effluents. The fathead minnow has a widespread distribution and is an important freshwater forage fish. It is also readily cultured in captivity. A large database on the effects of single chemicals has been developed using the fathead minnow for acute partial and life-cycle tests.

Modifications to the 32-day early life stages (ELS) test produced a 7-day larval growth and survival freshwater toxicity test. Norberg and Mount (1985) described this rapid method to assess the chronic toxicity of effluents using fathead minnows.

Healthy animals are the most important aspect for a good toxicity test. Emphasis should be placed on determining the quality of the organisms used for producing the test organisms. This report and the video *Culturing of Fathead Minnows* (*Pimephales promelas*) were produced by EPA to clarify and expand on culturing methods explained in the Acute Methods Manual (EPA, 2002a). To ensure successful toxicity testing, laboratory personnel should be familiar with the handling and culturing procedures detailed below.

The fathead minnow is an adaptable organism and can be cultured in the laboratory under a variety of conditions. Factors that must not be overlooked are the types of food to use for larvae and adults, the stocking rates to grow testing and breeding stock, and the water to use. For example, use of the brine shrimp (*Artemia*) as a basic food source has been essential at EPA-Duluth. Dried or processed fish foods such as the Purina Aquamax® trout food (formerly Trout Chow) can be very good as a dietary supplement, but few cultures have been successful using those diets alone. For cultures that require constant reproduction, the use of the fresh or frozen *Artemia* has been essential.

The density of the fish in the culture is very important. While fish can survive at high stocking densities, rapid growth, uniformity of size, sexual maturation, high reproduction rates, and limiting the spread of disease can be achieved with lower densities. With good diets, space, constant temperature and photoperiod, the fathead minnow can be cultured with ease. Keen observation and regular maintenance of the culture animals and conditions are essential for year-round organism production.

The first section of this report covers the selection of the culture water and explains procedures for natural, dechlorinated, and synthetic culture water preparation. The second section discusses food requirements and preparation. The third section explains procedures for initiating and maintaining fathead minnow cultures. The methods described in this report cover culturing requirements for fish for use in both acute and chronic tests, although the emphasis is primarily on generating animals for sublethal tests.

Culture Water Preparation and Delivery

The waters to be used for culturing fathead minnows are any toxicity-free freshwater including natural water, drinking water, or reconstituted water. The water source chosen for culturing may not necessarily be the same type of water used for testing. However, whichever water is chosen for culturing or testing, it must be tested to ensure that good survival and reproduction of the organisms are possible and that consistency is achievable. Before any water is used, it should be tested for possible contamination by pesticides, heavy metals, major anions and cations, total organic carbon, suspended solids, or any other suspected contaminants (OECD, 2006). The quality of the water should meet the acceptable levels described in Table 1. The water quality should ensure adequate survival, growth, and reproduction and it should be from a consistent source to provide constant quality during any given testing period.

Table 1. Recommended Chemical Characteristics of an Acceptable Culture Water (OECD, 1989)

Substance	*Concentration*
Particulate matter	< 20 mg/L
Total organic carbon	< 2 mg/L
Un-ionized ammonia	< 1 µg/L
Residual chlorine	< 10 µg/L
Total organophosphorus pesticides	< 50 ng/L
Total organochlorine pesticides plus Polychlorinated biphenyls	< 50 ng/L
Total organic chlorine	< 25 ng/L

Natural Water

Natural water can be from a variety of sources such as a surface water (e.g., river, lake, or pond), well water, or spring water. Natural waters should be carbon and/or sand filtered and fine filtered as well (~5μm). When using natural water that has resident fish populations, an ultraviolet sterilizer or ultrafilter may need to be added after the roughing filters to remove any potential fish pathogens.

Dechlorinated Water

Drinking water (i.e., city or tap water) may be used provided that it has received adequate treatment, but it may require dechlorination. This can be accomplished either by aeration for 24 hours or by using a carbon filter to remove residual chlorine. Sodium thiosulfate also may be used but it may act as a reducing/chelating agent in the water. The addition of 3.6 mg/L of anhydrous sodium thiosulfate will reduce 1.0 mg/L of chlorine. For fathead minnows, the 96-hour LC_{50} of sodium thiosulfate is 7.3 g/L (EPA, 1991). Following dechlorination, total residual chlorine should not exceed 0.01 mg/L. Because thiosulfate can be toxic to test organisms, a control lacking thiosulfate should be included for any toxicity tests utilizing thiosulfate-dechlorinated water (EPA, 2002b). Following dechlorination, tap water should be passed through a deionizer and carbon filter to remove metals and organics, and to control hardness and alkalinity.

Reconstituted Water

Two types of synthetic water are frequently used in testing, but the volumes required for culturing fish limit their application for culture. This is not to say that they are not suitable, but that their preparation is labor intensive, and the body of knowledge based on culturing in synthetic waters is small. Two types of synthetic fresh dilution water can be prepared. One is made using reagent grade chemicals and the other using a commercial mineral water. Both recipes are described in the Chronic Manual (EPA, 2002b). Recipes for preparing 20 liters of moderately hard water are given below.

The deionized water may be obtained from a Millipore Milli-Q®, QPak2® or equivalent system. Acceptable ranges for the physical/chemical characteristics of the dilution water are provided in Table 2. In order to extend the life of the Milli-Q® cartridges, use a preconditioned (deionized) feed water by using a Culligan®, Continental®, or equivalent system in front of the Millipore® system. In a four-cartridge Milli-Q® system place the cartridges in the order of (1) ion exchange, (2) carbon, (3) organic cleanup such as Organex-Q®, and (4) a final bacteria filter (0.22-μm fine filter). For a five cartridge system, add an additional carbon cartridge. The order of the filter heads may need to be re-plumbed so that the water flows over the cartridges correctly. Conductivity of this filtered water should be zero μmhos/cm. All filters should be changed at least every six months, but more frequent changes may be needed. The frequency of change is dependent on the source water.

Table 2. Water Quality Parameters for Reconstituted Waters (EPA, 2002b)

Water Type	*pH*	*Hardness**	*Alkalinity**
Very Soft	6.4 – 6.8	10 – 13	10 – 13
Soft	7.2 – 7.6	40 – 48	30 – 35
Moderately Hard	7.4 – 7.8	80 – 100	57 – 64
Hard	7.6 – 8.0	160 – 180	110 – 120
Very Hard	8.0 – 8.4	280 – 320	225 – 245

**Expressed as mg/l as $CaCO_3$*

The synthetic water made with reagent grade chemicals (Table 3) can be prepared in batches of 20 L using the following recipe:

- Place 19 L of Milli-Q® or equivalent water in a properly cleaned plastic carboy.
- Add 1.20 g of $MgSO_4$, 1.92 g of $NaHCO_3$, and 0.080 g of KCl.
- Aerate overnight
- Add 1.20 g of $CaSO_4 \cdot 2H_2O$ to 1 L of Milli-Q® or equivalent deionized water in a separate flask. Stir on a magnetic stirrer until the $CaSO_4$ is dissolved. Add to the 19 liters and mix well.
- Aerate vigorously for an additional 24 hours to dissolve the added chemicals and stabilize the medium.
- The measured pH, hardness, and alkalinity should be as listed in Table 2.

The synthetic water prepared using commercially available mineral water also can be prepared in large batches (Table 3). The instructions in this report are specifically for Perrier® water (EPA, 2002b). While other commercial waters have been tested, the properties of other waters have not been evaluated extensively; therefore no other commercial water instruction is provided. To prepare 20 L of water:

- Place 16 L of Milli-Q® or equivalent water in a properly cleaned carboy.
- Add 4 L of Perrier® water.
- Aerate vigorously for 24 hours to stabilize the medium.
- The measured pH, hardness, and alkalinity of the aerated water should be as listed in Table 4.
- This synthetic water prepared with Perrier® water is referred to as diluted mineral water (DMW) in toxicity test methods.

To aerate the water, use air free of oils and fumes. Organic vapors and oils can be removed using an in-line activated carbon filter such as Balston® C-1 (Balston, Inc., Lexington, MA.). Particles are removed using another in-line filter such as the Balston® Grade RX filter, used frequently in combination with the carbon filter.

Store both types of water in the carboys in which they were prepared and use each batch for only 14 days. Water should be stored away from direct light, and should be kept covered. Bacterial growth may occur in the water as it ages, which can cause problems for the culture organism.

Table 3. Preparation of Synthetic Fresh Water (EPA, 2002b)*

Type	Reagent Recipes				Mineral Water Recipes	
	Reagent Added (mg/l)				Vol. of Mineral Water Added (mL/L)	Proportion Mineral Water (%)
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_2$	KCl		
Very Soft	12.0	7.5	7.5	0.5	50	2.5
Soft	48.0	30.0	30.0	2.0	100	10.0
Moderately Hard	96.0	60.0	60.0	4.0	200	20.0
Hard	192.0	120.0	120.0	8.0	400	40.0
Very Hard	384.0	240.0	240.0	16.0	—	—

**Add reagent grade chemicals and/or mineral water to Milli-Q® or equivalent water.*

Table 4. Water Quality Parameters of Synthetic Freshwater Using Mineral Water (EPA, 2002b)

Water Type	pH	Hardness	Alkalinity
Very Soft	7.2 – 8.1	10 – 13	10 – 13
Soft	7.9 – 8.3	40 – 48	30 – 35
Moderately Hard	7.9 – 8.3	80 – 100	57 – 64
Hard	7.9 – 8.3	160 – 180	110 – 120
Very Hard	—	—	—

Water Delivery

If possible, a flow-through system should be used for culturing. The water delivery system should provide at least three to four turnovers per day. Threaded polyvinyl chloride (PVC) pipe is the most widely used construction material but glass, stainless steel, or Teflon® can be used. Rubber, copper, brass, or plastics containing fillers, additives, stabilizers, or plasticizers which may cause toxicity, and therefore, should not be used. Glued PVC should be used for drainage only, as the glue can be toxic.

Recirculating systems often consist of aquaria at table top level for the fish, and an aquarium on the floor that acts as a trickling filter for the drain water from the fish tanks. This filter can be made of any non-toxic, high surface area material like crushed coral, pea gravel, or specially designed plastic media for trickling filters. These are available through filtration suppliers. Nitrifying bacteria in these filters convert ammonia to nitrate when the system is in balance. Ammonia levels must be monitored closely in this type of system. Water can be pumped from a pump at the bottom of the filter up to a headbox above the fish tanks, where it flows back into the tanks.

Culturing fathead minnows is also possible using a static system. Each tank should have either an under-the-gravel or external filtration system. Supplies for these types of systems are available at hobby shops and aquarium supply houses. Every 2 to 3 days, renew the water by siphoning down at least 25% of the volume and adding new water. Use distilled water when replacing water lost due to evaporation to avoid concentrating the dissolved salts. An additional consideration is that larvae must be protected from being captured in the filtering system. A container made of fine mesh will allow water to flow through, while protecting the larvae.

For breeding, the aquariums can be divided into four chambers with stainless steel mesh. In flow-through and recirculating systems, each tank can be serviced by one water source, air stone, and drain.

Food Preparation

Fathead minnows are fed different forms of food during their development. From hatching to approximately 30 days their main diet is live brine shrimp (*Artemia salina*), and generally after one month old they are weaned over to partially frozen brine shrimp. The amount fed to each tank should be adjusted for the number and size of the fish.

Feeding The Larvae

Feed the larvae live brine shrimp twice each day, Monday through Friday, and once each day on weekends. Larvae require very small amounts of food during the first few days (days 1–5), but require increasing amounts on days 6–10. After 10 days, the feeding rate must increase substantially each day and is proportional to the number of fish maintained. Care must be taken not to overfeed during the first few days, and not to underfeed in the later stages. Careful observation is critical and waste food on the bottom of the tank indicates overfeeding. This decaying food will cause the dissolved oxygen (DO) levels to drop. Rapid consumption of all food right after feeding (i.e., all *Artemia* is consumed 5 – 10 minutes post feeding) indicates underfeeding. Shortage of food is also evident by wide size variability in 30-day old juvenile fish. The rotifers Brachionus spp. are an alternative food for the first feedings of larval fathead minnows.

Observe the amount of food left at the end of the day and adjust the feeding rate accordingly. Each day, siphon out any excess food as waste food will grow fungus that can trap the larvae.

Live Brine Shrimp

The brine shrimp used for feeding the larvae and juvenile fish is *Artemia salina*. Upon receiving brine shrimp cysts from a supplier, date the containers and store them in a freezer to prolong their shelf life.

Brine shrimp cysts are most easily hatched in containers with conical shaped bottoms or in separatory funnels. Typically hatching instructions are provided with the cysts. A common procedure is to make a 25 – 30 ppt salinity medium using un-iodized salt, and bubbling the water with filtered air from the bottom to keep the cysts circulating.

At 25° – 28°C, the *Artemia* begin to hatch in 24 hours. Larval fathead minnows must be fed less than 24-hour post-hatch *Artemia* nauplii so that they are small enough for the larvae to ingest. At this age, the nauplii also have their highest nutritional value as their yolk sacks have not yet been depleted. This nauplii size requirement makes it necessary to start new *Artemia* cultures daily.

To collect *Artemia* for feeding, remove the air supply and allow the unhatched cysts to settle to the bottom of the hatching jar (approximately 5 minutes). The live shrimp will settle forming an orange layer at the bottom of the container with a brown layer of unhatched cysts below it. The empty shells of the hatched cysts will rise to the top. The live shrimp can be removed using a large-bore pipette or a siphon. Either a 50-mL or 100-mL pipette (inverted) works well. Before feeding in static systems, or for static tests, rinse the *Artemia* with distilled, deionized, or culture water to prevent salt buildup in the tanks.

Frozen Brine Shrimp

Fish that are over 30 days old are fed partially frozen brine shrimp twice daily, Monday through Friday, and once a day on weekends. For easier handling, allow the brine shrimp to thaw slightly at room temperature (not completely). Each spawning pair should receive approximately ⅛–¼ teaspoon of the brine shrimp. As a general guide, feed each tank of fish the amount of food that can be consumed in about 10 – 20 minutes.

Nutritional quality and contaminant levels vary widely between strain, year of harvest, location of harvest, and supplier of *Artemia*. It is useful to get as much information as possible from the supplier concerning the nutritional quality and contamination or have the *Artemia* checked for these parameters as new batches are ordered.

Supplements

The periphyton that grows naturally in the tanks provides a good dietary supplement for the fish. In addition, flake food such as commercially available Tetramin® or Purina Aquamax® trout food (formerly Trout Chow) can be used as supplements.

Again, contaminant levels and nutritional content can vary widely, and screening and analysis may be required to ascertain the suitability of the foods.

Cultures

Initiating Cultures

Fathead minnow cultures should be started with fish from a reliable source such as a commercial supplier or a research laboratory. The embryos or fish should be shipped by overnight mail in an oxygenated container that is packed in a cooler to minimize temperature fluctuations. Upon receipt, allow the water in the shipping container to acclimate in a water bath or use aquarium heaters for the temperature adjustment. Once acclimated, empty the containers of starter cultures into a pan, aerate, and maintain the temperature at 25°C. In 4 – 5 days the embryos will hatch, at which time the larvae should be moved to rearing tanks using a large-bore pipette. For an 8- to 10-gallon tank, the recommended stocking density is 200–250.

Once embryos hatch, feed them *Artemia* salina nauplii that are less than 24 hours old. Feed the fish 2 to 3 times a day, 5 days a week, for at least the first 2 weeks. On weekends, feeding only once each day has proven to be adequate.

Breeding

When the fish are 30 days old, they are removed from rearing tanks to be grown out as brood stock. In a 50- to 70-gallon tank, 300 – 400 fish can be grown to maturity. For the brood stock fish, wean them over to frozen brine shrimp. To hasten the maturation process, thin the 3 – 4 month old fish to 30 – 35 per 10-gallon tank. The addition of spawning tiles may also speed up the maturation process.

Fathead minnows begin to show signs of maturity at three to four months of age (Figure 1). The male will develop an enlarged head with rows of tubercles across the snout. These are used to clean the underside of the spawning substrate on which the eggs are deposited. The male will also develop black coloration on his sides. The female is smaller than the male and will not have tubercles. She is an olivaceous color and when mature, exhibits an ovipositor.

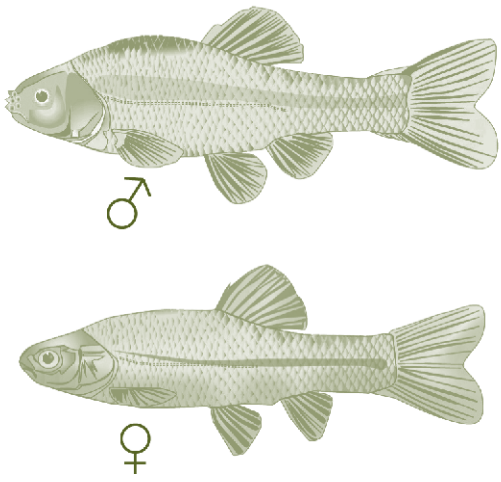

Figure 1. Male Fathead Minnow (top) and Female Fathead Minnow (bottom)

Spawning tiles can be made of clay tiles or 10 cm-diameter PVC pipes cut into 7 – 10 cm long sections which are cut in half lengthwise to create a semicircular arch. The inner side, where the eggs

will be deposited, is then roughened with sandpaper. When placed in the aquariums, the fish use the underside to deposit and fertilize the eggs. The rough surface helps the eggs to adhere to the substrate. In the wild, fathead minnows use the underside of submerged or floating objects.

Two options can be used for separating the fish for spawning. The first option separates the fish into spawning pairs. This is easily accomplished by dividing a 10- or 15-gallon aquarium into quarters using stainless steel screens. Paired spawning reduces competition which allows for greater production and makes it possible to monitor the fecundity of each pair. Daily records of reproduction can be used to identify sterile or spawned-out fish, which can be replaced by a new pair to maintain a high production rate.

A second breeding option is to place groups of mature adult fish in tanks with a female:male ratio of 8:3. Use four spawning substrates in an 8- to 10-gallon tank with approximately 20 fish. When the egg production rate in these tanks drops, replace the entire group of fish.

Spawning

The male cleans the underside of the spawning tile with his tubercles and draws the female underneath. The male directs her toward the tile where she releases the eggs. Fertilization of the eggs is external and the buoyant eggs stick to one another and adhere to the underside of the tile. Females release an average of 100 – 200 eggs per spawn, with larger females releasing 200 – 400 eggs. Fathead minnows spawn approximately every 4 – 5 days, but can spawn as often as every 2 days.

Monitor the reproduction rate of each brood pair or group of adults. If no embryos are produced in a 3-week period, replace the pair or the entire group. Younger fish can be allotted longer periods of time if they are just beginning to spawn.

Collecting The Embryos

The substrates in each tank should be checked daily for embryos. In order not to disrupt the early morning spawning, check the tanks midmorning. To retrieve the tiles, use tongs that are dipped in boiling water between each tank to minimize any possible transfer of disease from one tank to another.

If needed for toxicity testing, the embryos can be removed from the tile with a gentle, circular, rubbing motion while keeping the tile underwater to prevent premature hatching caused by the disruption. If the embryos are to be hatched directly from the tiles, transfer the tiles immediately to the hatching system.

Hatching

Two options for hatching the fathead minnow embryos are to remove the embryos from the tiles to aerated water in separatory funnels or to keep them on the tiles and hatch them in aerated water in larger pans. The first option requires that the embryos be rolled off of the tiles and pipetted into a separatory funnel containing aerating culture water. After 2 days in this system the embryos are placed in a pan containing aerated culture water. They will hatch in another 2 – 3 days.

The second option is to place the tiles directly into a pan containing aerating culture water in a holding pan. A white pan allows the larvae to be seen more easily. The tiles are placed on their sides covered with culture water, and aerated. At a temperature of 25°C, the embryos should hatch in 4 – 5 days.

The aeration of the water in the separatory funnel and around the tiles provides circulation and helps keep sediment and fungal spores from settling on the embryos. Check the embryos on the tiles on days 1 and 2 for fungus or lack of viability and remove any such embryos with tweezers. On days 3, 4, and 5, check the tiles but minimize any disturbances as it may cause early hatching of the larvae. Embryos that appear cloudy should be removed and discard all of the eggs from any tile on which 50% or more die. Figure 2 is a check list used at EPA-Duluth to track the daily tasks required for fathead minnow culturing.

After each use, the tiles are disinfected in a chlorine bath for 1 hour, rinsed with tap water, neutralized with sodium thiosulfate for at least 10 minutes to remove residual chlorine, and finally, rinsed in culture water and allowed to air dry.

Larvae for future brood stock should be the progeny of as many adult spawning fish as possible. A few larvae collected each month from many different spawning pairs will provide a broader gene pool than hundreds of larvae from one or two spawning pairs in one week.

Tracking The Fish

If the fathead minnows are used for toxicity testing there will be a need to anticipate the demand for the eggs or larvae. The number of eggs that are needed for testing will determine the number left to hatch for larval testing or to grow out for future brood stock. Figure 3 is an example of a request form used to anticipate the demand on the cultures. Figure 4 is a tracking form that EPA-Duluth uses to monitor the performance of their broods that are used for toxicity tests. It represents one method used to track the health of the cultures.

DAILY CHECK-LIST FOR FATHEAD CULTURE UNIT (Initial when done)												
DATE												
MORNING	Notes:											
Check temperatures												
Check water flow to all tanks												
Feed adult fish frozen brine shrimp												
Feed larvae live brine shrimp												
Check tiles in pans for bad eggs												
Pull spawning tiles and est. no. eggs (10:30)												
AFTERNOON												
Set up larvae												
Feed adult fish frozen brine shrimp												
Feed larvae live brine shrimp												
Check temperatures before leaving												

Figure 2. Suggested Daily Tasks for Fathead Culturing

EGG/LARVE REQUEST FORM					
Name	Date	Need (eggs or larvae)	How Many	When	Remarks

Figure 3. Testing Request Form

FATHEAD CULTURE UNIT: Fish Tracking Form	
Date:	
Researcher:	
Age of Fish (Embryos, Larvae?):	
Number Taken:	
Type of Test (Embryo-Larval, 7 day, etc.):	
Test Conditions (Static, Renewal, Flow-Through?)	
Temperature:	
Dilution Water:	
Test Chemical:	
Hatching Percentage (Embryos):	
Control Survival (%) (Reps. Different?):	
Abnormal Survival in Low Concentrations?:	
Deformities in Control Fish?:	
Observations (i.e., Condition of Fish, Test Conditions):	
RETURN FORM TO FATHEAD CULTURE UNIT BEFORE INITIATING NEXT TEST!	

Figure 4. Performance Tracking Form

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Glossary

Artemia. The marine invertebrate (referred to as brine shrimp) used as the recommended food source for culturing fathead minnows. Brazilian or Columbian strains are preferred because the supplies are found to have low concentrations of chemical residues.

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

Nauplii. Free-swimming microscopic larvae stage characteristic of copepods, ostracods, barnacles, etc. typically only with three pairs of appendages.

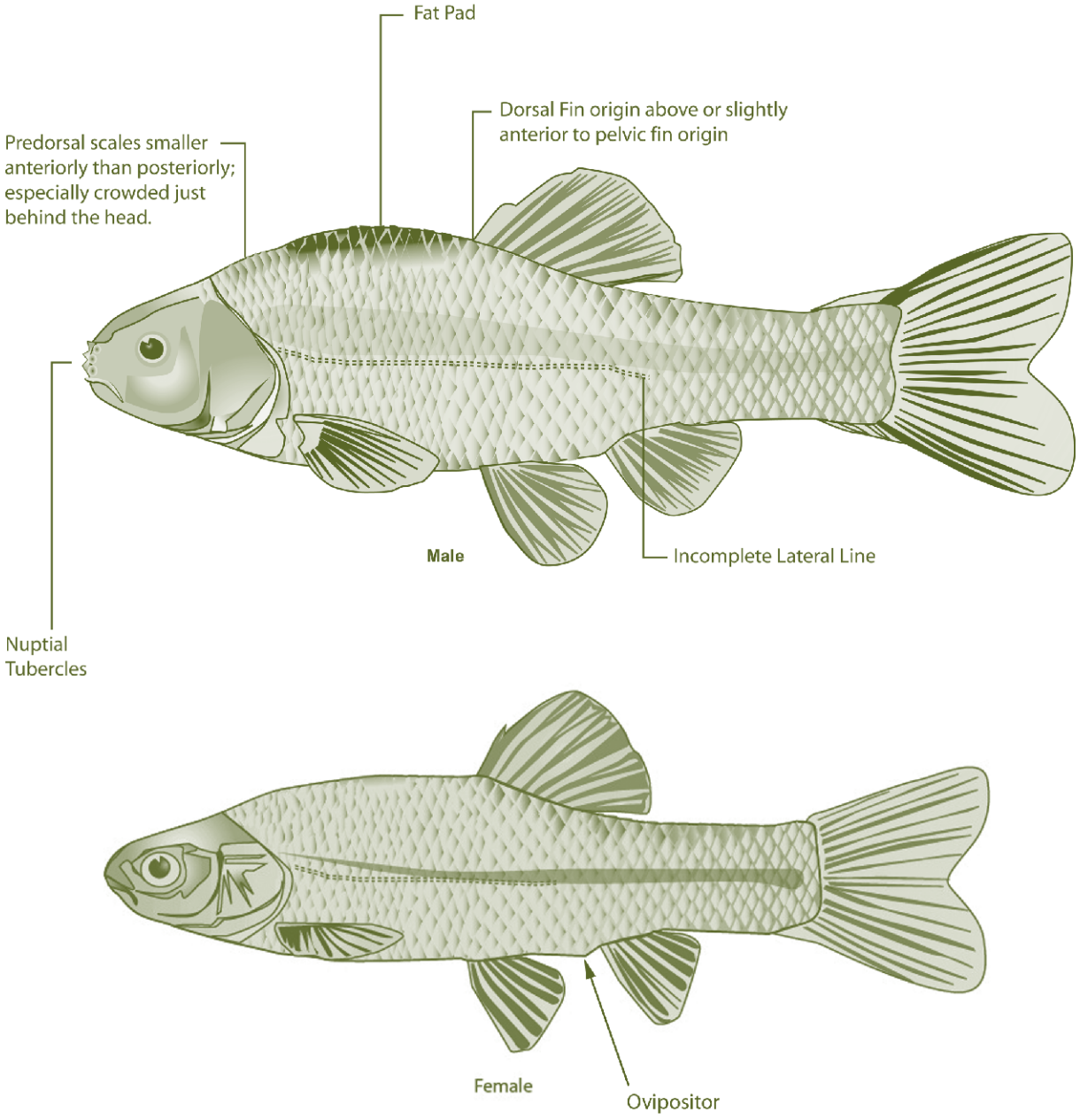
Ovipositor. The tubular extension of the female pore in certain fishes used to assist in depositing eggs.

Pimephales promelas. Scientific name for the fathead minnow, a common freshwater vertebrate minnow of the family Cyprinidae which is widely distributed east of the Rockies.

Tubercles. Spongy protrusions on the dorsal surface of the male fish, anterior to the dorsal fin; these are used by the male to clean the debris from spawning substrate and fertilized embryos.

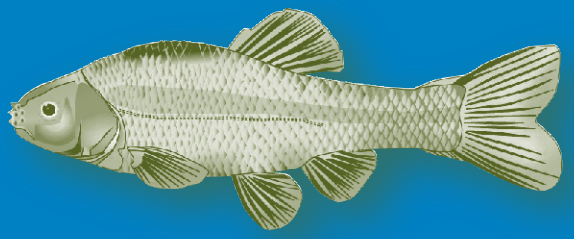
Appendix A:

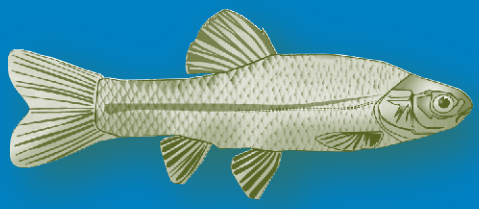
Illustration of Fathead Minnow with Anatomical Identifications



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

Supplement to Training Video





U.S. Environmental Protection Agency
Office of Wastewater Management
Water Permits Division
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Washington, DC 20460

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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Contents

TABLE

FIGURE

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).

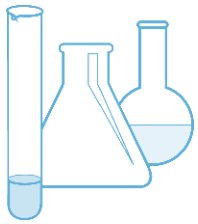
Section 8 of the Chronic Manual covers sample collection. Note that surface waters should be filtered (60 µm plankton net) for fathead minnow tests.

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.

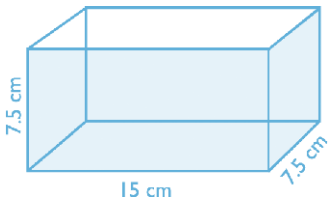
Dissolved oxygen, temperature, pH, and total residual chlorine must be measured on each new sample. EPA also recommends that total alkalinity, total hardness, and conductivity be measured on each new sample. Dissolved oxygen, temperature, and pH are measured at the beginning and end of each 24 hour renewal 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.

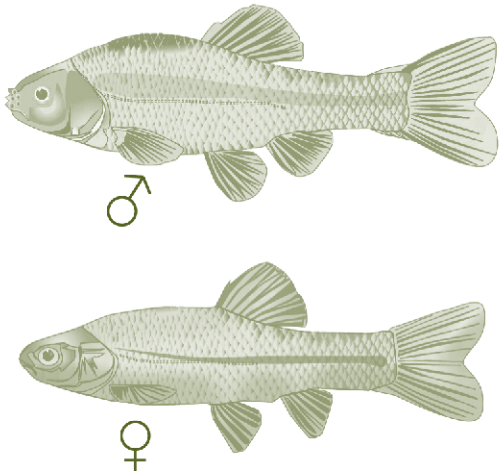


Figure 1. Male Fathead Minnow (top) and Female Fathead Minnow (bottom)

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).

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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

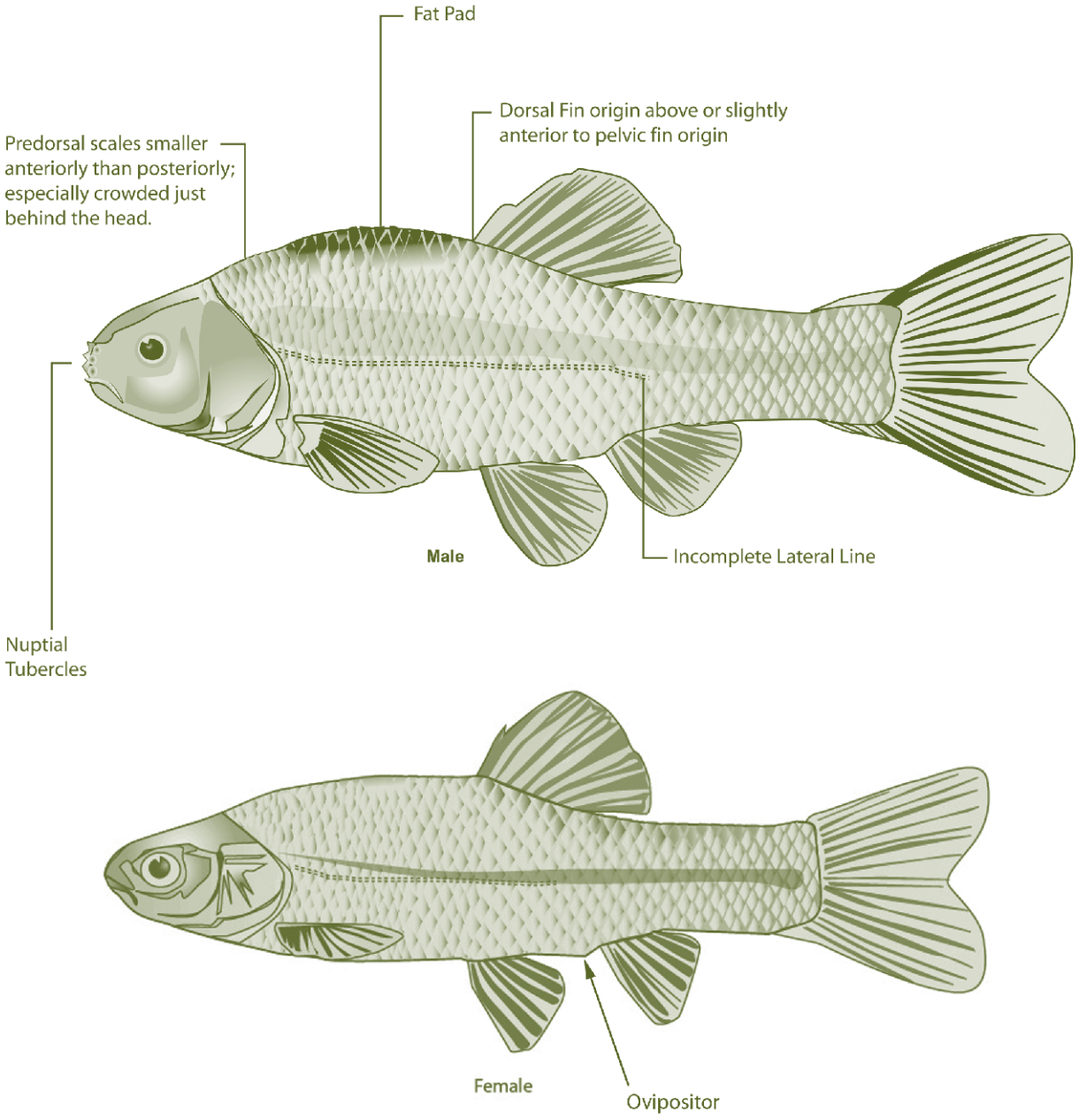
Summary of Test Conditions and Test Acceptability Criteria for Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Toxicity Tests with Effluents and Receiving Waters (Test Method 1000.0)

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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