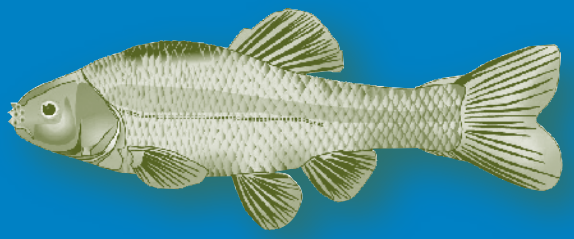
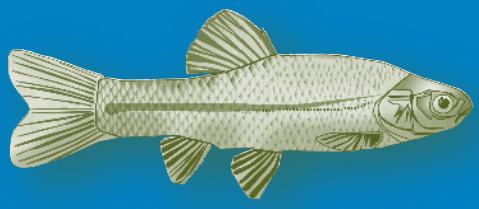
Culturing of Fathead Minnows (*Pimephales promelas*)

Supplement to Training Video





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NOTICE

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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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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.

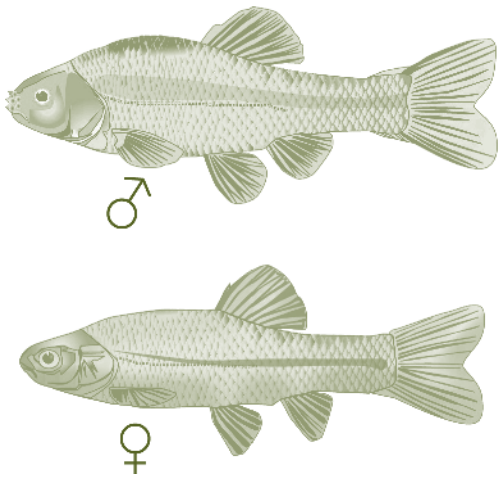

Figure 1. Male Fathead Minnow (top) and Female Fathead Minnow (bottom)

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

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