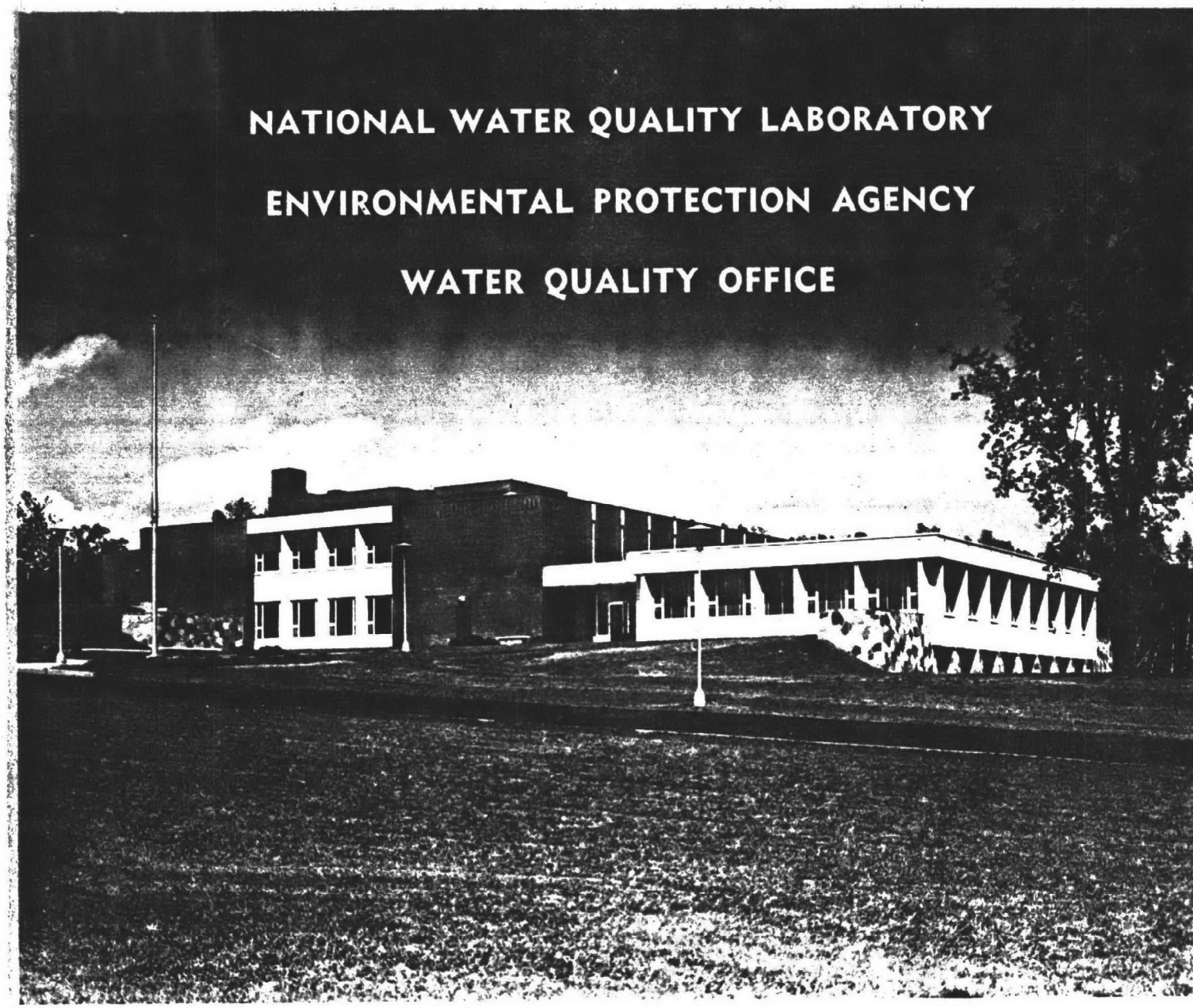


# PROPOSED BIOASSAY PROCEDURE FOR

# FATHEAD MINNOW PIMEPHALES PROMELAS RAFINESQUE CHRONIC TESTS

(Revised January, 1972)

# PROPOSED BIOASSAY PROCEDURES

## Preface

Proposed Bioassay Procedures are established by the approval of both the Committee on Aquatic Bioassays and the Director of the National Water Quality Laboratory. The main reasons for establishing them are: (1) to permit direct comparison of test results, (2) to encourage the use of the best procedures available, and (3) to encourage uniformity. These procedures should be used by National Water Quality Laboratory personnel whenever possible, unless there is a good reason for using some other procedure.

Proposed Bioassay Procedures consider the basic elements that are believed to be important in obtaining reliable and reproducible results in laboratory bioassays. An attempt has been made to adopt the best acceptable procedures based on current evidence and opinion, although it is recognized that alternative procedures may be adequate. Improvements in the procedures are bing considered and tested, and revisions will be made when necessary. Comments and suggestions are encouraged.

Committee on Aquatic Bioassays

Director, National Water Quality Laboratory

Proposed Bioassay Procedure for

Fathead Minnow Pimephales promelas Rafinesque Chronic Tests

(Revised January, 1972)

## A. Physical system

1. Diluter: Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or indirectly, through measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter or water supply failure.

2. Toxicant mixing: A container to promote mixing of toxicant bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check at least once every month to see that the intended amounts of water are going to each duplicate tank or chamber.

3. Tank: Two arrangements of test tanks (glass, or stainless steel with glass ends) can be utilized:

   a. Duplicate spawning tanks measuring 1 x 1 x 3 ft. long with a one sq. ft. portion at one end screened off and divided in half for the progeny. Test water is to be delivered separately to the larval and spawning chambers of each tank, with about one-third the water volume going to the former chamber as to the latter.

   b. Duplicate spawning tanks measuring 1 x 1 x 2 ft. long with a separate duplicate progeny tank for each spawning tank. The larval tank for each spawning tank should be a minimum of 1 cu. ft. dimensionally and divided to form two separate larval chambers with separate standpipes, or separate 1/2 sq. ft. tanks may be used. Test water is to be supplied by delivery tubes from the mixing cells described in Step 2 above.

   Test water depth in tanks and chambers for both a & b above should be 6 inches.

4. Flow rate: The flow rate to each chamber (larval or adult) should be equal to 6 to 10 tank volumes/24 hr.

5. Aeration: Total dissolved oxygen levels should never be allowed to drop below 60% of saturation, and flow rates must be increased if oxygen levels do drop below 60%. As a first alternative flow rates in the spawning tanks can be increased above those specified in A.4. Aerate with oil free air only if testing a non-volatile toxic agent and then only as a last resort to maintain dissolved oxygen at 60% of saturation.

6. Cleaning: All adult tanks, and larvae tanks and chambers after larvae swim-up, must be siphoned a minimum of 2 times weekly and brushed or scraped when algal or fungus growth becomes excessive.

7. Spawning substrate: Use spawning substrates made from inverted cement and asbestos halved, 3-inch ID drain tile, or the equivalent, each of these being 3 inches long.

8. Egg cup: Egg incubation cups are made from either 3-inch sections of 2-inch OD (1 1/2-inch ID) diameter polyethylene water hose or 4-oz., 2-inch OD round glass jars with the bottoms cut off. One end of the jar or hose sections is covered with stainless steel or nylon screen (with a minimum of 40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968). The vertical-travel distance of the cups should be 1 to 1 1/2 inches.

9. Photoperiod: The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month. The table is arranged so that adjustments need be made only in the dusk times. Regardless of the actual date that the experiment is started, the Evansville test photoperiod should be adjusted so that the mean or estimated hatching date of the fish used to start the experiment corresponds to the Evansville test day-length for December first. Also, the dawn and dusk times listed in the table need not correspond to the actual times where the experiment is being conducted. To illustrate these points, an experiment started with 5-day-old larvae in Duluth, Minnesota, on August 28 (actual date), would require use of a December 5 Evansville test photoperiod, and the lights could go on anytime on that day just so long as they remained on for 10 hours and 45 minutes. Ten days later (Sept. 7 actual date, Dec. 15 Evansville test date) the day-length

would be changed to 10 hours and 30 minutes. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), if desired, should be included within the day-lengths shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

10. Temperature: Temperature should not deviate instantaneously from 25° C by more than 2° C and should not remain outside the range of 24 to 26° C for more than 48 hours at a time. Temperature should be recorded continuously.

11. Disturbance: Adults and larvae should be shielded from disturbances such as people continually walking past the chambers, or from extraneous lights that might alter the intended photoperiod.

12. Toxic materials: Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. It is best to avoid such problems when possible, rather than to try to overcome them through chemical measurements. Even though what is significant will vary from test to test and most materials sorb and/or leach small amounts of at least some substances, some generalizations are possible. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very signficant amounts of trace organic compounds.

    The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

B. Biological system

1. Test animals: If possible, use stocks of fathead minnows from the National Water Quality Laboratory in Duluth, Minnesota or the Fish Toxicology Laboratory in Newtown, Ohio. Groups of starting fish should contain a mixture of approximately equal numbers of eggs or larvae from at least three different females.

Set aside enough eggs or larvae at the start of the test to supply an adequate number of fish for the acute mortality bioassays used in determining application factors.

2. Beginning test: In beginning the test, distribute 40 to 50 eggs or 1 to 5-day-old larvae per duplicate tank using a random assignment. All acute mortality tests should be conducted when the fish are 2 to 3 months old. If eggs or 1 to 5-day-old larvae are not available, fish up to 30 days of age may be used to start the test. If fish between 20 and 60 days old are used, the exposure should be designated a partial chronic test. Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see B.12.) or for residue analysis (see C.4.).

3. Food: Feed the fish a frozen trout pellet (e.g., Oregon Moist). A minimum of once daily fish should be fed ad libitum the largest pellet they will take. Diets should be supplemented weekly with live or frozen-live food (e.g., Daphnia, chopped earthworms, fresh or frozen brine shrimp, etc.). Larvae should be fed a fine trout starter a minimum of 2 times daily, ad libitum; one feeding each day of live young zooplankton from mixed cultures of small copepods, rotifers, and protozoans is highly recommended. Live food is especially important when larvae are just beginning to feed, or about 8 to 10 days after egg deposition. Each batch of food should be checked for pesticides (including DDT, TDE, dieldrin, lindane, methoxychlor, endrin, aldrin, BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

4. Disease: Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

5. Measuring fish: Measure total lengths of all starting fish at 30 and 60 days by the photographic method used by McKim and Benoit (1971). Larvae or juveniles are transferred to a glass box containing 1 inch of test water. Fish should be moved to and from this box in a water-filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light platform to provide background illumination. Photos are then taken of the fish over

the millimeter grid and are enlarged into 8 by 10 inch prints. The length of each fish is subsequently determined by comparing it to the grid. Keep lengths of discarded fish separate from those of fish that are to be kept.

6. Thinning: When the starting fish are sixty (± 1 or 2) days old, impartially reduce the number of surviving fish in each tank to 15. Obviously injured or crippled individuals may be discarded before the selection so long as the number is not reduced below 15; be sure to record the number of deformed fish discarded from each tank. As a last resort in obtaining 15 fish per tank, 1 or 2 fish may be selected for transfer from one duplicate to the other. Place five spawning tiles in each duplicate tank, separated fairly widely to reduce interactions between male fish guarding them. One should also be able to look under tiles from the end of the tanks. During the spawning period, sexually maturing males must be removed at weekly intervals so there are no more than four per tank. An effort should be made not to remove those males having well established territories under tiles where recent spawnings have occurred.

7. Removing eggs: Remove eggs from spawning tiles starting at 12:00 noon Evansville test time (Appendix A) each day. As indicated in Step A.9., the test time need not correspond to the actual time where the test is being conducted. Eggs are loosened from the spawning tiles and at the same time separated from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the eggs being to roll. The groups of eggs should then be washed into separate, appropriately marked containers and subsequently handled (counted, selected for incubation, or discarded) as soon as possible after all eggs have been removed and the spawning tiles put back into the test tanks. All egg batches must be checked initially for different stages of development. If it is determined that there is more than one distinct stage of development present, then each stage must be considered as one spawning and handled separately as described in Step B.8.

8. Egg incubation and larval selection: Impartially select 50 unbroken eggs from spawnings of 50 eggs or more and place them in an egg incubator cup for determining viability and hatchability. Count the remaining eggs and discard them. Viability and hatchability determinations must be made on each spawning (>49 eggs) until the number of spawnings (>49 eggs) in each duplicate tank equals the

number of females in that tank. Subsequently, only eggs from every third spawning (>49 eggs) and none of those obtained on weekends need be set up to determine hatchability; however, weekend spawns must still be removed from tiles and the eggs counted. If unforseen problems are encountered in determining egg viability and hatchability, additional spawnings should be sampled before switching to the setting up of eggs from every third spawning. Every day record the live and dead eggs in the incubator cups, remove the dead ones, and clean the cup screens. Total numbers of eggs accounted for should always add up to within two of 50 or the entire batch is to be discarded. When larvae begin to hatch, generally after 4 to 6 days, they should not be handled again or removed from the egg-cups until all have hatched. Then, if enough are still alive, 40 of these are eligible to be transferred immediately to a larval test chamber. Those individuals selected out to bring the number kept to 40 should be chosen impartially. Entire egg-cup-groups not used for survival and growth studies should be counted and discarded.

9. Progeny transfer: Additional important information on hatchability and larval survival is to be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or larvae, and by transferring eggs from these concentrations to the control tanks. One larval chamber in, or corresponding to, each adult tank should always be reserved for eggs produced in that tank.

10. Larval exposure: From early spawnings in each duplicate tank, use the larvae hatched in the egg incubator cups (Step B.8. above) for 30 or 60 day growth and survival exposures in the larval chambers. Plan ahead in setting up eggs for hatchability so that a new group of larvae is ready to be tested for 30 or 60 days as soon as possible after the previously tested group comes out of the larval chambers. Record mortalities, and measure total lengths of larvae at 30 and, if they are kept, 60 days post-hatch. At the time the larval test is terminated they should also be weighed. No fish (larvae, juveniles, or adults) should be fed within 24 hr's. of when they are to be weighed.

11. Parental termination: Parental fish testing should be terminated when, during the receding day-length photoperiod, a one week period passes in which no spawning occurs in any of the tanks. Measure total lengths and weights of parental fish; check sex and condition of gonads. The gonads of most parental fish will have begun to regress from the spawning condition, and thus the differences between the sexes will be less distinct now than previously. Males and females that are readily distinguishable from one another because of their external characteristics should be selected initially for determining how to differentiate between testes and ovaries. One of the more obvious external characteristics of females that have spawned is an extended, transparent anal canal (urinogenital papilla). The gonads of both sexes will be located just ventral to the kidneys. The ovaries of the females at this time will appear transparent, but perhaps containing some yellow pigment, coarsely granular, and larger than testes. The testes of males will appear as slender, slightly milkly, and very finely granular strands. Fish must not be frozen before making these examinations. Use discarded fish for tissue residue analysis (skin, bone, muscle, gill, brain, liver, kidney, GI tract, and gonad should be considered) and physiological measurements of toxicant related effects.

12. Special examinations: Fish and eggs obtained from the test should be considered for physiological, biochemical, histological and other examinations which may indicate certain toxicant related effects.

13. Necessary data: Data that must be reported for each tank of a chronic test are:

    a. Number and individual total length of normal and deformed fish at 30 and 60 days; total length, weight and number of either sex, both normal and deformed, at end of test.

    b. Mortality during the test.

    c. Number of spawns and eggs.

    d. Hatchability.

    e. Fry survival, growth, and deformities.

C. Chemical system

1. Preparing a stock solution: If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

   Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose, only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxyethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxyethanol, $OPE_{10}$) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

   The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. TL50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

2. Measurement of toxicant concentration: As a minimum the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentration of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3. Measurement of other variables: Temperature must be recorded continuously (see A.10.).

   Dissolved oxygen must be measured in the tanks daily, at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

   A control and one test concentration must be analyzed weekly for pH, alkalinity, hardness, acidity, and conductance or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are affected by the toxicant the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis so that each tank is analyzed once every other week.

   At a minimum, the test water must be analyzed at the beginning and near the middle of the test for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

4. Residue analysis: When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

5. Methods: When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: Use true duplicates for each level of toxic agent, i.e., no water connections between duplicate tanks.

2. Distribution of tanks: The tanks should be assigned to locations by stratified random assignment (random assignment of one tank for each level of toxic agent in a row followed by random assignment of the second tank for each level of toxic agent in another or an extension of the same row).

3. Distribution of test organisms: The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.). At time of thinning (B.4.) the choice of males and females must also be made randomly.

E. Miscellaneous

1. Additional information: All routine bioassay flow through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater, (American Public Health Association, 1971), or information requested from appropriate persons at Duluth or Newtown.

2. Acknowledgments: These procedures for the fathead minnow were compiled by John Eaton for the Committee on Aquatic Bioassays. The participating members of this committee are: Robert Andrew, John Arthur, Duane Benoit, Gerald Bouck, William Brungs, Gary Chapman, John Eaton, John Hale, Kenneth Hokanson, James McKim, Quentin Pickering, Wesley Smith, Charles Stephan, and James Tucker.

3. References: For additional information concerning flow through bioassays with fathead minnows, the following references are listed:

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Approved by the Committee
on Aquatic Bioassays

Approved by the Director, NWQL

## Appendix A

### Test (Evansville, Indiana) Photoperiod

### For Fathead Minnow Full Chronic

| Dawn to Dusk Time | Date | | Day-length (hour and minute) | |
|---|---|---|---|---|
| 6:00 - 4:45) | DEC. | 1 | 10:45) | |
| 6:00 - 4:30) | | 15 | 10:30) | |
| | | | ) | |
| 6:00 - 4:30) | JAN. | 1 | 10:30) | |
| 6:00 - 4:45) | | 15 | 10:45) | |
| | | | ) | |
| 6:00 - 5:15) | FEB. | 1 | 11:15) | 5-month pre- |
| 6:00 - 5:45) | | 15 | 11:45) | spawning growth |
| | | | ) | period |
| 6:00 - 6:15) | MAR. | 1 | 12:15) | |
| 6:00 - 7:00) | | 15 | 13:00) | |
| | | | ) | |
| 6:00 - 7:30) | APR. | 1 | 13:30) | |
| 6:00 - 8:15) | | 15 | 14:15) | |
| | | | | |
| 6:00 - 8:45) | MAY | 1 | 14:45) | |
| 6:00 - 9:15) | | 15 | 15:15) | |
| | | | ) | |
| 6:00 - 9:30) | JUNE | 1 | 15:30) | |
| 6:00 - 9:45) | | 15 | 15:45) | 4-month spawning |
| | | | ) | period |
| 6:00 - 9:45) | JULY | 1 | 15:45) | |
| 6:00 - 9:30) | | 15 | 15:30) | |
| | | | ) | |
| 6:00 - 9:00) | AUG. | 1 | 15:00) | |
| 6:00 - 8:30) | | 15 | 14:30) | |
| | | | | |
| 6:00 - 8:00) | SEPT. | 1 | 14:00) | |
| 6:00 - 7:30) | | 15 | 13:30) | |
| | | | ) | |
| 6:00 - 6:45) | OCT. | 1 | 12:45) | post spawning period |
| 6:00 - 6:15) | | 15 | 12:15) | |
| | | | ) | |
| 6:00 - 5:30) | NOV. | 1 | 11:30) | |
| 6:00 - 5:00) | | 15 | 11:00) | |