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GUIDELINES FOR CONDUCTING EARLY LIFE STAGE TOXICITY TESTS WITH JAPANESE MEDAKA (ORYZIAS LATIPES)

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FOREWORD

In recent years the Japanese Medaka, Oryzias latipes, has become an important test animal for use in evaluating carcinogenesis. A review of the literature by Couch and Harshbarger (1) revealed that the medaka are known to be quite susceptible to some classes of chemical carcinogens. Hawkins, et al. (2) have also demonstrated the development of chemically induced tumors in several medaka organs such as the liver, kidney, eye and muscle. Because of the medaka's apparent sensitivity to chemically induced tumor development, its short life cycle time, rapid growth and well known culture techniques, the U.S. Environmental Protection Agency's Environmental Research Laboratory in Duluth, Minnesota recently began a medaka chemical carcinogen screening program. The USEPA at Duluth is also currently evaluating the medaka for use in developing freshwater aquatic life criteria documents.

This procedural guide is based upon evaluations of published papers and recent methods development work conducted at the U.S. EPA laboratory in Duluth, Minnesota. The purpose of this report is to provide information and basic guidelines for conducting an early life stage toxicity and/or carcinogen test with medaka. This test starts with 24 hour old embryos and ends 4 weeks after hatch. The guideline also describes recently developed methods for shortening the hatch period and methods for incubating embryos without the use of chemical treatments to control fungus. It is also the intention of the authors to make these procedures compatible with ASTM's Annual Book of ASTM Standards: Guide for Conducting Early Life-Stage Toxicity Tests with Fishes, (Volume 11.04; designation E1241-88). The report has been reviewed by the Environmental Research Laboratory-Duluth, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

This manual represents a procedural guide for conducting embryo-larval early life stage (ELS) toxicity tests with Japanese medaka (Oryzias latipes). These procedures are based upon evaluation of published papers and recent methods development work conducted at our laboratory in Duluth. The published papers are referenced in the appropriate places throughout the text of this report. If more detailed information on test apparatus or specific biological and chemical methods is desired, the reader is encouraged to study the reference material or contact one of the authors of this manual. All routine methods not covered in this procedure (physical and chemical determinations), should be followed as described in Standard Methods for the Examination of Water and Wastewater (3). Those routines dealing with handling of fish not covered in this procedure should be followed as described by ASTM (4).

CHAPTER 1

PHYSICAL SYSTEM

1.1 Diluter

Intermittent-flow proportional diluters (5, 6) or continuous-flow serial diluters (7, 8) should be used. The operation of the diluter should be checked daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations with a dilution factor not greater than 0.50 and a control should be used for each test.

1.2 Toxicant Mixing and Flow Splitting

If a proportional diluter is used, a container to promote mixing toxicant and diluent water should be used between the diluter and test tanks for each concentration (9). Separate flow splitter delivery tubes should run from this container to each replicate tank. If a continuous-flow serial diluter is used, additional mixing containers are not needed, but separate flow splitter delivery tubes must run from the diluter to all test tanks. Delivery tubes are allocated to tanks by stratified random assignment (random assignment of one delivery tube for each level of concentration in a row followed by random assignment of the duplicate delivery tube for each level of concentration in the other row). Flow splitting accuracy should be within 10% and should be checked periodically to see that the intended amount of test water reaches each tank.

1.3 Test Tanks

All test tanks should be made either of glass or stainless steel. Glass is usually preferred because it is less expensive. Typically, duplicate test tanks have been arranged in two rows back to back with test concentrations assigned by stratified random assignment. Many sizes of test tanks have been used successfully. Any tank size, shape and depth is acceptable if the flow

rates and loading requirements can be achieved. One small test tank which has been used routinely for medaka ELS tests is described as follows:
Up to sixty medaka (hatch-1 month old) have been tested in each tank (duplicate tanks per treatment) that measured 133 x 178 x 127 mm high containing 2000 mL of test water with a water depth of 85 mm and a flow rate of 33 ml/minute. Water levels were controlled by a side drain in the end of each tank. Side drains were covered with a removable 40 mesh (40 openings per inch) cylindrical stainless steel screen to prevent loss of 1-10 day old fish after which a 20 mesh cylindrical stainless steel screen was used to prevent loss of 11-28 day old fish. It is also recommended that a circulating water bath system be used with the test tanks so that temperature differences between tanks can be held to within $\pm$ 0.5 °C.

1.4 Embryo Incubation Cups

Embryo incubation cups (Figure 1) are made from 20 ml glass scintillation vials with the bottom portion cut away leaving a 4.5 cm upper portion to form the cup. Sixty mesh Nitex screen is glued over the 1.5 cm ID mouth of the vial. The bottomless vial is then inverted and a # 5 1/2 neoprene rubber stopper with a 1 cm hole bored through it is inserted into the cut end of the vial (2.5 cm ID). A 20 gauge stainless steel wire loop handle about 3.5 cm high is shaped and inserted into the upper end of the stopper. During embryo incubation the small cups are oscillated vertically 2.5-4.0 cm in the test water by means of a rocker arm apparatus driven by a 10 rpm motor (Figure 2). The combination of this size motor and cup configuration causes each embryo to move up and down in the vial with a cascading rolling motion. Recent studies at our laboratory have shown that this method of embryo incubation for medaka greatly shortens the time to hatch period (mean test days to complete hatch @ 28°C = 7.5; mean hatch = 96%). The method also significantly reduces the number of fungused embryos throughout the incubation period as compared to the embryo incubation system used for fathead minnows which utilizes a 2 rpm motor

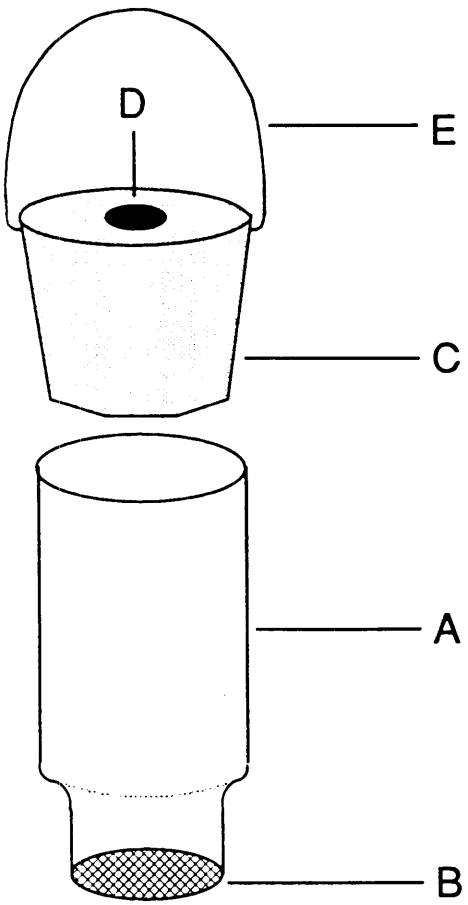

Figure 1. Medaka embryo incubation cup made from a 20 ml glass scintillation vial (A) with 60 mesh Nitex screen (B) #5.5 rubber stopper (C) with a 1 cm hole (D) and wire handle (E).

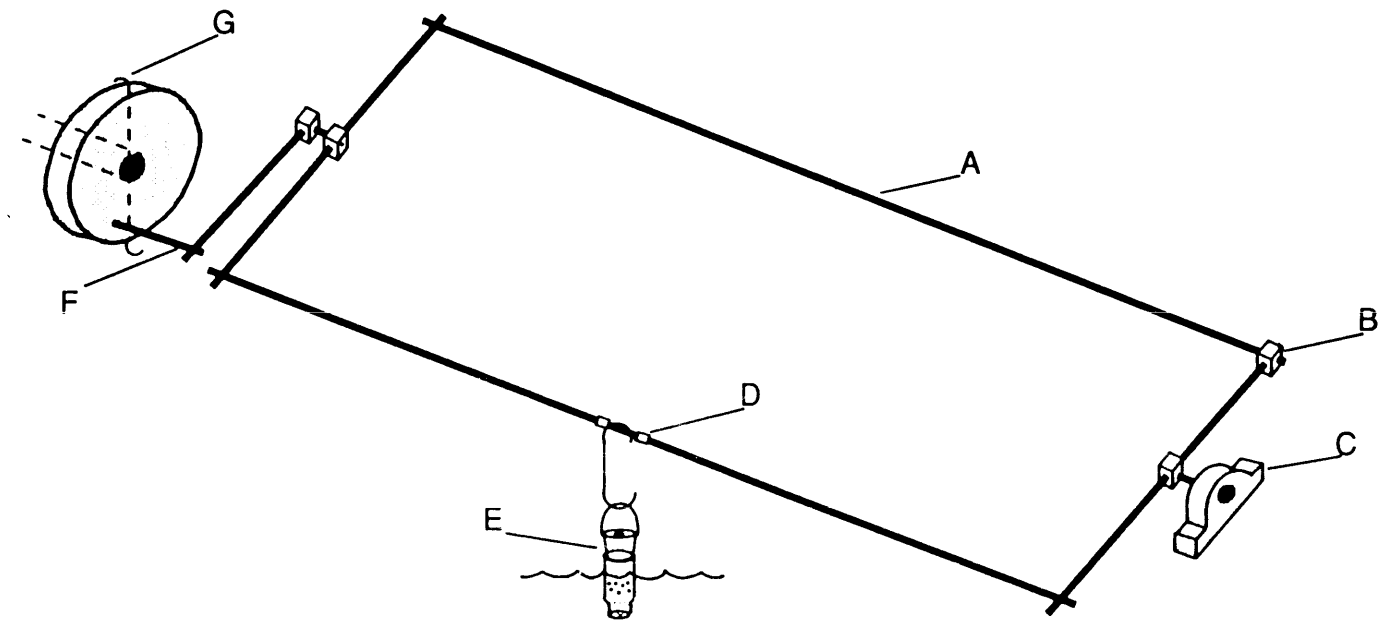

Figure 2. Rocker arm assembly for medaka embryo incubation and hatch. (A) rocker frame made from 1/2" aluminum Flexaframe rod (Fisher), (B) 90° Flexaframe connector on each corner and end supports, (C) 1/2" pillow block support bearing on each end, (D) hose clamp on each side of wire hanger to keep cup in place, (E) incubation cup suspended in each test tank on both sides of rocker frame, (F) connector rod attached with bolt on each end so that the rod will rotate freely up and down as stopper turns, (G) wire inserted through #11 stopper and shaft of 10 rpm motor to secure stopper to shaft. (10 rpm motor, model 10BF4209UAS, Blan Eletronics Corp., 52 Warren St., NY. NY., 10007)

drive (10). These studies have also shown no increase in larval mortality and/or deformities at hatch.

1.5 Dilution Water

The water used should be from a well or spring, if at all possible, or from a surface water source (4). Only as a last resort should dechlorinated water from a municipal water supply be used.

1.6 Loading and Flow Rate

The loading in test tanks should not exceed 0.1 gram of fish per liter of test water passing through the tanks in 24 hours (4). Flow rates to larger test tanks (10-20 liter) should be at least 6-10 water volumes per 24 hours. Flow rates, to the smaller test tanks (0.5 - 2.0 liter) should be at least 20-24 volumes per 24 hours. During a test, flow rates should not vary more than 10% between any tanks. Flow rates should be great enough so that dissolved oxygen does not drop below 60% of saturation (4) or toxicant concentrations drop by more than 20% when fish are in the test tanks. Flow can be increased above those specified rates to maintain proper dissolved oxygen or toxicant concentrations. With a continuous flow diluter system delivering one tank volume every hour, oxygen levels can be maintained above 75% in 2 liter volume tanks containing 60 four week old medaka.

1.7 Aeration

Diluent water should be aerated vigorously (with oil-free air) or passed over a screen column with a recirculating pump before flowing through the diluter. Aeration of diluent water will eliminate supersaturation of dissolved gases and also ensure that dissolved oxygen concentrations will be at or near saturation (90-100%). However, the test tanks themselves should not be aerated (aeration can alter toxicity by driving of volatile chemicals and it is also difficult to aerate all tanks at exactly the same rate).

1.8 Test Water Temperature

Test water temperature should not deviate from 28°C by more than ±1°C and should not remain outside the range of 26 to 30°C for more than 48 hours at a time. At a minimum, temperature should be measured in one tank at each toxicant concentration once a week, alternating between duplicate tanks from week to week. Temperature should also be recorded daily in one control tank.

1.9 Photoperiod and Lighting

A 16-hour light and 8-hour dark photoperiod controlled by an automatic timer should be used throughout the test in order to provide long daylight feeding hours and to promote normal maturation. A 15-30 minute transition period between light and dark is optional (11). Cool white fluorescent tubes have been used successfully for embryo-larval tests and light intensities at the water surface have ranged from 20-60 lumens (12). The intensity selected should be duplicated as closely as possible for all test tanks. Another option for lighting is a 50:50 combination of Durotest (Optima FS) and wide spectrum Grow-Lux fluorescent tubes.

1.10 Cleaning

Incubation cup screen bottoms should be cleaned periodically after embryos are removed. Screens can usually be cleaned with tap water pressure, and/or a small brush. After hatch is complete and larvae swim-up all tanks should be carefully siphoned at least three times a week and scraped if algal or fungal growth becomes noticeable. Siphoning should be done just before the last feeding of the day.

Siphoning can be done safely with a large pipette (50 ml) fitted with a squeeze bulb or with a siphon hose leading to a white dishpan. Fish which are siphoned accidentally can be observed easily in the pipette or white pan and returned carefully to the tank without harm.

1.11 Disturbance

Fish should be shielded from excessive disturbances such as people walking past the tanks or extraneous lights that might alter the intended photoperiod.

1.12 Construction Materials

Construction materials which contact the diluent water should not contain leachable substances and should not absorb significant amounts of substances from the water (4). Stainless steel and glass are the preferred construction materials. Rubber and plastics containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents are not known to contain leachable materials, nor do they absorb significant amounts of test substances. All batches of neoprene stoppers should be checked for toxicity prior to use in the diluter and exposure tanks. Static tests at the U.S. EPA, Environmental Research Laboratory-Duluth, have shown that certain lots of neoprene stoppers are acutely toxic to fathead minnow larvae (S.J. Broderius, personal communication, U.S. Environmental Protection Agency, Duluth MN 55804).

CHAPTER 2

CHEMICAL SYSTEM

2.1 Preparing a Stock Solution

Distilled or diluent water should be used in making up the test stock solutions. The development of several chemical saturators for use with hydrophobic chemicals has eliminated the need to use carrier solvents with most test chemicals (13-17).

If carrier solvents other than water are absolutely necessary, they should be of reagent grade or better but the amounts used should be kept to a minimum. Triethylene glycol (TEG) and dimethyl formamide (DMF) are preferred, but methanol, ethanol or acetone can also be used. The calculated solvent concentration to which any test organisms are exposed should never exceed 0.1 ml/liter.

When a carrier is used, use two sets of duplicate controls. One set should contain no solvent and one set should contain the highest concentration of solvent to which any organism in the test is exposed (4).

2.2 Measurement of Toxicant Concentration

At a minimum, the concentration of toxicant in the test water should be measured in one replicate at each toxicant concentration every week, alternating weekly between replicates. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include surface film or material stirred up from the bottom or sides of the tank.

Methods described in Methods for Chemical Analysis of Water and Wastes (18) and Manual of Analytical Methods for the Analysis of Pesticides in Human and Environmental Samples (19) should be used when possible. Accuracy should be

measured using the method of known additions for all analytical methods for toxicants. Reference samples should be analyzed periodically for each analytical method.

2.3 Measurement of Other Variables

Dissolved oxygen and pH should be measured at each concentration at least once a week, alternating weekly between replicates.

A control, high and median test concentration should be analyzed at least initially for alkalinity, hardness, conductance and turbidity to show the variability in the test water. If any of these characteristics is affected by the toxicant, that characteristic should be measured at each concentration at least once a week and alternated between replicates. Alkalinity, hardness, conductance and turbidity should be measured at least once a week in alternate control replicates for the duration of the test.

2.4 Residue Analysis

After the ELS test is complete all surviving fish may be analyzed for toxicant residues. Additional samples for analyzing toxicant residues can be measured during the test, if desired, to determine the toxicant uptake curve. The frequency and amount of samples taken will depend on the test objectives. Since medaka usually are consumed whole by predators, whole body residues should be reported.

CHAPTER 3

BIOLOGICAL SYSTEM

3.1 Source of Test Fish

Sufficient numbers of embryos can be transported by express from a brood stock culture unit to other laboratories or field sites to initiate an early life stage test. These embryos should be shipped in well-oxygenated water in insulated containers.

It is, however, recommended that a laboratory brood stock culture unit be started at your facility by obtaining embryos from another well-established culture unit such as is maintained at the Environmental Research Laboratory in Duluth MN. Guidelines for the culture of several life stages of the medaka can be obtained from the Environmental Research Laboratory in Duluth, MN (20).

At 27-28°C and a constant 16-hour day light photoperiod, fish fed unrestricted quantities of live brine shrimp nauplii will mature in 3-4 months. With proper care and maintenance these adult fish will produce quality embryos for 6-8 months (20).

3.2 Preliminary Tests

Selection of ELS test concentrations, a critical part of the experiment, should be made using the utmost judgment and care. All embryos or larvae used in preliminary and ELS tests should be taken from the same adult stock. As a general rule, one should conduct a small scale 96-hour static range finder test (five concentrations plus control) with ten 1-2 day old larvae in each of six 250 ml beakers with 200 ml of test water in each beaker. Test beakers should be covered (i.e., with parafilm) to slow evaporation and to reduce the loss of volatile chemicals. Larvae need not be fed during the 96-hour test. The selection of range finder concentrations (based upon previous toxicity information and/or saturation concentration) should be spread out by as much

as a factor of 10 depending on how much is known about the test chemical. If the chemical in question is highly volatile and/or more than 20% is lost during the range finder, then the test may have to be conducted as a renewal test or possibly even as a flow-through test. Throughout the 96-hour test, all fish should be carefully observed so that not only mortality is noted but also any behavioral effects such as abnormal swimming, surfacing, nervous movements, or lethargy. Concentrations which produce definite behavioral effects during the 96-hour larval test will usually cause significant survival and/or growth effects during the longer ELS test.

Depending on how distinct the effects are from the range finder test, the investigator may then either set up another static preliminary test with a dilution factor of 0.5 or conduct a small scale 10-12 day flow-through exploratory test (0.5 dilution factor, five concentrations plus control) with ten 1-2 day old larvae (fed live brine shrimp). Either test should be based upon the most sensitive indicator of stress observed during the range finder test.

If mortality was the only observed effect during preliminary testing, then the highest test concentration selected for the ELS test should be no less than the 96-hour LC20 and no more than the 96-hour LC50 calculated from the preliminary tests. If distinct behavioral effects were the most sensitive indicators of stress then the highest test concentration selected for the ELS test should be equal to that concentration.

3.3 Obtaining Embryos

It is desirable to have a medaka culture unit located on site so that the cultures can more easily be managed to provide adequate embryos at the proper time. Two days before the ELS test is to be started all spawning sponges (20) in the culture unit are removed and cleared of embryos. Since medaka nearly always spawn during the early daylight hours (21), these sponges should be

cleared in the afternoon. Some females may still be carrying their egg clutch from the morning spawning but to prevent injury to the brood stock it is not recommended that these clutches be removed.

Maximum numbers of similar age medaka embryos (<24 hours old) can be obtained for testing if the sponges are not replaced in the spawning tanks until the very late evening hours (shortly before photoperiod controlled lights go out) on the day before embryos are to be collected. By that time all of the females carrying clutches from the previous day will have dropped them and most of the females spawning during the early morning hours of that day will also have dropped them. By using this technique, about 85-95% of the embryos collected the following afternoon are less than 24 hours old.

The following afternoon all sponges with embryos are removed from the spawning tank and suspended in a shallow white dishpan in flowing water (~50 ml/min @ 27-28°C) (20). A rough estimate of total number of embryos is also made at this time to determine if there are enough to start an ELS test (20). The embryos are left to develop on the sponges overnight, and are removed the next morning to start the test. This important step helps to reduce fungus growth during embryo incubation. With the aid of a dissecting scope, 24 hour old viable embryos (neurula stage) can easily be staged and counted. Nonviable eggs and fungused, opaque or abnormal appearing embryos, as well as 48 hour old embryos (optic lens and cup stage) laid by females which were still carrying egg clutches when the spawning sponges were replaced in the tanks, can also be identified and discarded. It has been our experience that medaka embryo viability can be expected to be 95-98%.

Prior to staging each group of embryos, the long sticky filaments are removed from the chorion of each embryo as described in the following section. If filaments are not removed, embryos will be difficult to separate when staging

and will clump together in the embryo incubation cup which will cause fungus to grow on them before hatch.

3.4 Embryo Filament Removal

Recent methods development work conducted at our laboratory has provided a simple apparatus and technique for the fast and safe removal of sticky filaments from each embryo. The "embryo roller" apparatus is made from a wide mouth 270 ml glass jar (72 mm OD) with four evenly spaced 5 mm deep notches cut into the top, and the bottom cut off and replaced with soft Nitex screen (approximately 90 mesh) attached with silicone glue (Figure 3). The modified jar is then inverted with the notched mouth side down and placed in the bottom portion of a glass petri dish (100 x 20 mm).

Embryos (200-300) from about 10-12 sponges at a time are gently picked off by hand (index finger and thumb) and placed in a small round-bottomed glass or stainless steel bowl containing diluent water maintained at ~28°C. Embryos are then gently swirled around in the bowl. When the swirling motion slows down, embryos can easily be siphoned up from the center of the bowl with a 50 ml pipette and squeeze bulb (pipette tip cut off and a 7 mm OD glass tube inserted and glued to the inside of the pipette tube). Embryos are then carefully transferred from the pipette to the 90 mesh screen on the embryo rolling apparatus. Water transferred with the embryos will drain through the screen and into the petri dish below. Embryos are then rolled around on the screen for about two minutes with a gentle circular motion of the index finger while pressing on them lightly. The motion and pressure of the finger is almost identical to that described by Gast and Brungs (22) for removing fathead minnow embryos from spawning substrates. The rolling action of the embryos on the screen causes the filaments to begin sticking to one another which in turn causes the filaments to begin breaking off from each rolling embryo. Loose filaments form a sticky ball which quickly adheres to other attached filaments and breaks them off. This method, developed for filament

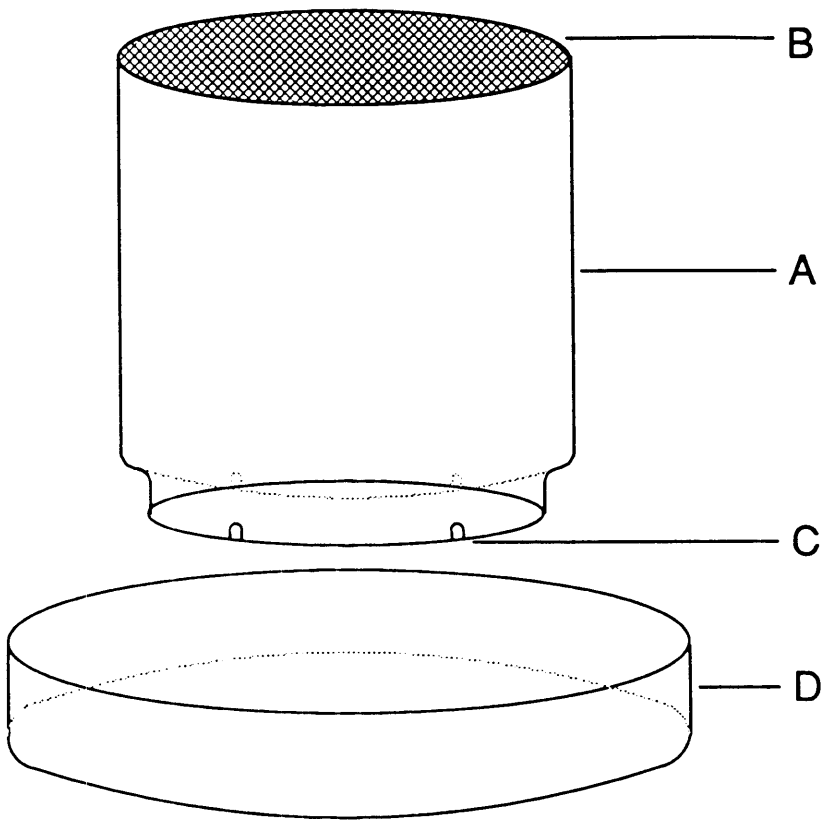

Figure 3. Medaka embryo roller for removal of long sticky filaments. Embryo roller is made from a wide mouth 270 ml glass jar (A) with 90 mesh Nitex screen (B) drainage notches (C) and a glass petri dish (D) placed underneath.

removal, has been shown to have no adverse effects on either embryo development or hatch.

Embryos with filaments removed are then transferred for staging by simply turning the embryo roller over and immersing embryos on the screen into a water filled petri dish (100 x 20 mm). Filament balls can be easily picked out of the petri dish with tweezers and discarded. After each group of 200-300 embryos is staged, they should be pooled in a small round-bottomed bowl containing 28°C diluent water.

3.5 Embryo Exposure (test day 0-9)

The test is started by counting and distributing a minimum of 40 embryos (~24 hours old) with a large bore eye dropper to one incubation cup standing in 27-28°C diluent water and then randomly assigning that cup to a replicate test tank. This process is repeated until all replicates have one incubation cup suspended on the moving rocker arm apparatus.

Because of the medaka embryo's tendency to attract fungus rapidly, it is necessary to gently flush out each group daily from the incubation cup into a glass petri dish. Embryos are then examined with a dissecting scope and dead or fungused embryos counted and discarded. Embryos can easily be flushed out by inverting the cup at a sharp angle over the petri dish and slowly pouring 27-28°C diluent water through the bottom of the cup screen. The cup should then be examined under a magnifier viewer light to make sure that all embryos are out of the cup. Early fungus development can be more easily observed on the embryos if an index card is placed over the stage light of the dissecting scope so that the embryos can be viewed over the light along the edge of the index card. The defracted light helps to make the early stages of fungus development more visible.

On test day 4 the embryos will be well developed and eyed-up (23); therefore, on this day each group should be randomly reduced to a minimum of 20 per cup after they are flushed out for their daily examination. This number may vary depending upon the number of fish needed for examination at the end of the test.

3.6 Embryo Hatch (test day 5-9).

With an incubation temperature of 28°C, embryos (with sticky filaments removed) will normally begin to hatch on the fifth day of the test. It has been our experience that most embryos that are incubated as described in section 1.4 will hatch on days 6 and 7 of the test.

Hatched larvae from each cup should be counted daily after they are flushed into the petri dish. Live fish that appear lethargic or show abnormal swimming behavior or physical appearance should also be noted at this time, but should not be discarded. All live larvae are then released into the test tank, dead larvae are discarded and unhatched embryos returned to the cups after inspection under the dissecting scope for fungal growth. Embryos not hatched after ten days of exposure should be counted as dead and discarded. It might, however, be necessary to extend the incubation period for a few days if it is suspected that the toxicant is causing a delayed hatch. The average exposure days-to-hatch (to the nearest day) in each cup should also be recorded.

Recent medaka experiments at our laboratory, using the above embryo handling techniques, have demonstrated that one can expect the normal mean percent hatch to be about 95% (minimum 80%; maximum 100%); and the average normal test days to complete hatch to be about 7.5 days (minimum 7; maximum 9). It has also been determined upon close examination that those embryos which have not hatched by test day 10 are either dead or in such a weakened condition that they are unable to hatch. Further studies using the above methods by the

authors have also shown that normal larval mortality and/or deformities at hatch were consistently low, (mean = 0.3%, minimum 0%; maximum 2%).

3.7 Larval Exposure (mean hatch test day 7 - final test day 35).

Feeding methods for the medaka are generally the same as those used for the fathead minnow (24). Larvae should be fed equal quantities of live brine shrimp nauplii (e.g., Bio-Marine, San Francisco Bay, or Jungle Brand) within 1-2 days after hatching. Measured volumes of the nauplii in a concentrated freshwater slurry are dispensed to each replicate with a small pipette (3-5 ml) fitted with a squeeze bulb. Quantities fed should be increased weekly as the fish grow larger in order to insure that adequate food is available throughout the daylight hours. The slurry must be mixed well between replicate feedings to insure uniformity of volumes fed. Feeding schedules are generally either twice a day about six hours apart or three times a day at least four hours apart. Fish may, however, be fed double rations once a day on weekends and holidays. Studies completed by the authors have shown that brine shrimp are quite hardy and can remain alive for up to 5-6 hours in fresh water. Each new lot of brine shrimp eggs should be analyzed for presence of pesticides, before use.

Dead fish should be counted and removed daily throughout the exposure period. Sometimes larvae which die (especially during the first 2 weeks after hatch) deteriorate so rapidly in the warm water that they cannot be easily observed in the test tank; therefore, the final count of live fish at the end of the test is used to calculate survival.

On exposure day 35 (28 days after mean hatch day), surviving fish in each replicate are killed by placing them on ice for several minutes. The wet weight of each individually blotted fish is then determined to the nearest milligram. Total length also may be reported if desired. Make note of any lethargic or any fish exhibiting abnormal movements, swimming behavior or

physical appearance. Fish should not be fed during the last 24 hours of the exposure period.

If fish are to be transferred to clean water for further studies such as residue half-live studies or tumor development research, live fish can be weighed as follows. Individual fish are blotted on paper toweling through a small net and then are carefully dropped from the net into a container of water which has been tared to zero on a balance. The balance is tared to zero after each fish is weighed. If desired, total lengths of live fish can be determined photographically as described by McKim and Benoit (25). An early life-stage test with medaka is generally considered to be unacceptable when the overall average survival in any control tank is less than 70%.

Recent medaka experiments at our laboratory using the above larval handling techniques have demonstrated that one can expect the normal mean survival from embryo thinning (test day 4) to 28 days after hatch to be about 90% (minimum 80%; maximum 98%). Results from these experiments also demonstrated that the normal mean weight measured 28 days after hatch was 55 mg per fish (minimum - 47 mg; maximum 72 mg) with a standard deviation of $\pm$ 7 mg.

3.8 Calculation of Results

The primary data to be analyzed from the medaka early life-stage test are those on (a) percent survival in each replicate (may be analyzed as embryo survival, larval survival and/or overall survival), (b) mean weight of individual survivors in each replicate, and (c) mean chemical concentration of the test solution in each treatment. Depending on the individual test objectives and results, additional analyses may also be done to determine toxicant effects on embryos, hatchability, deformities, total lengths and other chemical factors such as residue uptake and measurements on temperature, oxygen, pH, etc.

The variety of procedures that can be used to calculate results of a medaka early-life stage test can be divided into two categories: those which test hypotheses, and those which provide point estimates (4). The calculation procedures and interpretation of results must be appropriate to the experimental design of the test. Alternative methods and points to be considered when selecting and using procedures for calculating results of early-life stage toxicity tests are discussed by ASTM (4).

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