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DRAFT STUDY PLAN

COMPARATIVE EVALUATION OF FATHEAD MINNOW ASSAYS FOR DETECTING ENDOCRINE-DISRUPTING CHEMICALS

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PREPARED FOR

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1.0 PURPOSE OF STUDY

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) composed of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. Including the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ enough from those on higher vertebrates that mammalian screening methods may not identify potential endocrine-disrupting chemicals in this class of animals.

U.S. EPA (2001) has described a short-term test with the fathead minnow that considers reproductive fitness as an integrated measure of toxicant effects and enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-] estrogens and androgens. The test is initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed; and fecundity and fertilization success are monitored. At selected times during the test, embryo hatching and larval survivability are measured. At termination of the test, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin and sex steroids (17β-estradiol, testosterone, 11-ketotestosterone).

The purpose of work conducted under this task is twofold: (1) evaluate the transferability and sensitivity of short-term reproduction tests with the fathead minnow to identify specific modes of action of endocrine disruptors using four model compounds, and (2) conduct a side-by-side comparison of the 21-day fathead minnow short-term reproduction test (EPA 2001) to two separate 14-day assays: one a shortened version of the 21-day assay with less intensive monitoring of reproductive performance and the other an assay using pre-spawning fathead minnows (OECD Draft 31 December 2001). This latter assay is simplified by not measuring reproductive performance parameters.

2.0 ENDPONTS OF INTEREST

Survival

Daily assessment of survival is necessary to provide a basis for expressing and interpreting reproductive output, that is, number of eggs/female/d. Unless unacceptable water quality excursions and/or disease occur, it is rare to observe mortality in untreated control animals during the chemical exposure phase of the test. In fish exposed to the test chemical, overt lethality may occur, particularly in later portions of the assay not reflective of the initial (shorter) range-finding test. This has been a problem in the past with some test agents such as methyl-testosterone (Ankley et al. 2001).

Behavior of Adults

General observations on spawning behavior can be made for fathead minnows. Quantitative observation requires considerable effort whether those observations are made directly by individuals or through the use of video equipment. Special consideration must be made to ensure that the observation process does not impact the true spawning behavior of the fish. The collection of eggs occurs at the same time and is also very labor-intensive. Therefore, labor constraints at this time of the study may limit the amount of quantitative data that can be collected.

Secondary Sex Characteristics

Secondary sex characteristics in fish are hormonally controlled, making them viable endpoints for evaluating endocrine disruption. The male fathead minnow in particular has distinct breeding tubercles on the snout and a dorsally located fat pad.

It is important to identify how the observation and measurement of secondary sex characteristics will be used. General observation or qualitative results can be made easily for the fathead minnow. Qualitative results would provide supportive evidence of endocrine disruption and would be useful in a screening test. Quantitative analysis also can be performed on semi-empirical observations of secondary sex attributes in chemically exposed fish.

Fecundity

Fecundity can be measured by counting the number of laid eggs and spawns from spawning groups of fish. Because the general spawning strategy for fish is to produce many eggs with limited or no parental protection, a large number of eggs can be produced by a female in a relatively short time. The collection of eggs is a very time-consuming phase in the study, especially if the eggs are to be evaluated for fertilization or hatching success.

More variability is seen in spawning data compared with data endpoints, such as fertilization success, hatching success, length, weight, and survival. Because of the limitations of replication, other endpoints might be more sensitive than fecundity when measured based on the number of eggs deposited over time. Because fathead minnows are fractional spawners, the time to deposit eggs might be the most critical endpoint. For example, the number of eggs-per-female-per-day over a two- to three-week period might be similar, but higher treatment levels might induce spawning over a shorter time. However, if eggs are released from the ovary before they are mature, fertilization success might also be affected.

Fertilization Success

In addition to the formation of adequate numbers of gametes for reproduction, it is also important that the gametes possess high viability for successful fertilization and larval survivability. Egg viability can be affected in a number of ways, including reduced Vitellogenin (VTG) incorporation into the oocyte causing smaller sized eggs or disturbances in the levels of sex and thyroid hormones (reviewed in Kime & Nash 1999). Fertilization success and sperm

viability is often correlated with sperm motility (Lahnsteiner et al. 1998). Screening assays that expose both genders simultaneously and then determine fertilization success will effectively measure the combined viability of eggs and sperm.

Hatchability, Larvae Appearance, and Survival

The formation of the zygote is the beginning of the embryonic period in animal life history. The embryonic period for fishes ends with the transition from the endogenous yolk supply to exogenous feeding (Balon 1975). In oviparous fishes such as fathead minnows, the embryonic period will encompass all development within the egg and can extend after hatching until the yolk supply is exhausted. This is advantageous for the study of endocrine disruptors as the eggs are released by the female and develop external to the parents. Therefore, they can be easily studied after spawning and fertilization without invasive procedures on the adults. Also, the early development is rapid, as a consequence of their small size, short lifecycle, and preference for warm water temperatures.

Gonad Size (GSI) and Morphology, and Biochemical Endpoints (VTG, steroids)

Gonadosomatic Index

The GSI is a general measure of the growth status of reproductive tissues. Tissue-somatic indices are commonly reported in fisheries studies because of the relative ease of determination and the general belief that certain indices, such as the liver-somatic index, can be an excellent predictor of adverse health in fish (Adams & McLean 1985). The GSI is also frequently reported as a general measure of gonad maturation and spawning readiness and is based on the broad assumption that proportionally larger gonads indicate greater development (West 1990).

The GSI can be potentially useful as part of a reproductive screen because reduction in relative gonad mass can occur as a response to certain types of endocrine-active compounds. The general procedure for determining the GSI is simple and involves humanely euthanizing the fish, removing excess moisture and determining the total mass, and then removing and weighing the gonads. The index is then calculated as GSI = 100 × gonad weight / body weight. Typical values for reproductively active fathead minnows are: females 8% to 12%; males ~1% (Jensen et al. 2001).

Gonad Morphology

Histopathology continues to be widely applied in assays for the effects of xeno-estrogens and other chemical agents (Legler et al. 2000; Wester & Canton 1986; Wester & Canton 1990). Histological methods are widely practiced for assessment of morphology. General histological methods are relatively routine. A protocol for both paraffin-embedded and glycol-methacrylate-embedded histology was described by Jensen et al. (2001). Paraffin embedment is a longer established and more commonly practiced method of embedding, adapted to processing high volumes of tissue samples.

A standard chemical fixative should be used for evaluations, since choice of fixative can affect degree of tissue shrinkage and tinctorial or staining properties of the tissues. The use of routine fixatives such as neutral buffered formalin, Bouin's fixative, and buffered glutaraldehyde-formaldehyde for fixating fish reproductive tissues has been reported (Jensen et al. 2001; Schwaiger et al. 2000; Miles-Richardson et al. 1999b; Wester & Canton 1986). Tissues should be placed in the fixative immediately after excision from specimens, and adequate fixation time should be allowed prior to initiating infiltration and embedment procedures. Typically, sections are stained with hematoxylin and eosin to observe tissue and organ structures.

To evaluate the gonads of fathead minnows, sections should be taken along the long axis of the gonad at 4- to 5-μm intervals in a serial-step fashion. Multiple sections are desirable, and standardized procedures for producing such multiple sections need to be established for any protocol. More precise details are provided by Panter et al. 2001. For histopathological analysis to be especially useful in reproductive screening tests, it is important to ensure maximum control of the subjective nature of the interpretations and to limit the interpretation to repeatable results that can be verified by different investigators. Toward this end, the following steps should be implemented:

- Staging and abnormalities are best defined by incremental changes (i.e., presence or absence of particular structures) and, in all cases, need to codified in a standardized definition that can be used by multiple investigators.
- Measurement of the size of structures is subject to a high degree of measurement error as a result of a plane of section variations and must, therefore, be compared using appropriate statistical tests.
- Methods for fixating, embedding, sectioning, and staining need to be standardized.
- Unknown samples should be read and interpreted in a blind fashion.

Biochemical Endpoints

Vitellogenin

Vitellogenin is an egg-yolk precursor protein that is synthesized in the liver of fish prior to its transport to the ovaries and incorporation into developing oocytes. Synthesis of VTG is under estrogen control, mediated by estrogen receptors (ER) in the liver. Both male and female fish can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen mimics such as methoxychlor. Detection of VTG has become the most widely studied biomarker of exposure to endocrine-active compounds. In many fish, induction of VTG has been shown to be extremely sensitive to estrogen exposure.

Tissue Steroid Concentrations

The measurement of plasma levels of E2, 11-KT, and T can be used as endpoints to assess sex-steroid status in male and female fish, provided there is some knowledge about normal population levels when the samples are taken. In the context of a laboratory toxicology

experiment, a control group of sufficient size is an absolute requirement. Plasma levels of E2 have limited utility in males, because levels of this steroid are usually low or non-detectable. The measurement of plasma E2 is most useful in sexually maturing females because of the gradual rise in this hormone during the period of vitellogenesis. In many fish, E2 begins to decline by the time of final maturation, probably as a consequence of the completion of yolk synthesis by the ovary, and E2 levels drop significantly after that. A distinct switch is seen in fish ovarian steroidogenic pathways from estrogens to progestins at this time (Nagahama 1999).

Similar to E2 in female fishes, 11-KT is the sex steroid characteristic of the sexually maturing male. Very low levels (e.g., pg/mL range) are sometimes reported in adult female fish (Jensen et al. 2001; Simpson & Wright 1977), although the physiological significance, if any, is not understood. In toxicology studies, 11-KT is measured in the blood of male fish to assess androgen status, with the expectation being that reduced levels of this hormone are synonymous with reproductive dysfunction. This correlation has not been conclusively established, although the necessity of 11-KT for fish spermatogenesis has emerged (Miura et al. 1991; Schulz et al. 2001). Testosterone levels are normally much lower than 11-KT in males at the time of spawning (Jensen et al. 2001). However, plasma levels of T can approach E2 levels in females of some fish, such as the fathead minnow (Jensen et al. 2001), and would also be a useful endpoint to measure in assessing the endocrine status of sexually mature fish.

An important concern with single measurements of plasma sex steroids in mature fish is the relevance to reproductive dysfunction. It is now well documented that circulating levels of sex steroids can vary seasonally and, in the case of fathead minnows, on a daily basis during the spawning cycle (Jensen et al. 2001). This would be expected to increase variability in the measurement of these parameters from groups of asynchronous spawning fish. An additional confounding problem is high inter-laboratory variability in the measurement of plasma steroids (McMaster et al. 2001), making it difficult to determine subtle effects on steroid levels and, hence, reducing the sensitivity of the endpoint. As an aid in determining mode of action, steroid ratios, specifically E2 / 11-KT or E2 / T in females, may be more useful. This approach was used to analyze steroid measurements made in carp collected from various sites in the United States (Goodbred et al. 1997). The basis for this analysis is the hypothesis that sex steroid ratios, as opposed to their absolute values, are more important in determining sexual differentiation and, perhaps, sexual development (Jalabert et al. 2000).

3.0 DOSE SELECTION

The test concentrations to be used in this assignment are summarized below.

Test Chemical	Exposure Concentration (µg/L)		
	Low	Mid*	High
Fadrozole	2.0	25	50
Methoxychlor	1.0	2.5	5.0
Methyl Testosterone	50	125	200
Flutamide	0.06	0.35	0.65

*Mid concentration is only tested in the OECD 14-day test.

4.0 STUDY PROTOCOLS

4.1 Short-term (21-day) Reproduction Assay with the Fathead Minnow for Identification of Endocrine-Disrupting Chemicals

Introduction

This protocol describes the short-term reproduction assay recently developed by Ankley et al. (2001) using the fathead minnow *(Pimephales promelas)*. This short-term assay measures the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints are performed to aid identification of the specific toxicological mode of action of the assay chemical.

The assay is started with mature male and female fish previously evaluated for spawning activity. During the subsequent 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed, along with daily measurements of fecundity and fertilization success. Successful embryological development is measured based on the hatching success of fertilized eggs. At termination of the assay, measurements are made of external morphology, GSI, gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT).

Principle of the Assay

An overview of the assay and relevant test conditions is provided in Table 1. The assay is initiated with mature adults that have a record of reproductive success as measured both by fecundity (number of eggs) and embryo viability (e.g., hatchability). This is established during a "pre-exposure" period of 14 to 21 days in the same system/tanks as will be utilized for the chemical test. The assay is conducted at a minimum of two chemical concentrations, as well as appropriate controls, with four experimental units (replicates) per treatment. Each replicate tank contains four female and two male fish. The test chemical is delivered to the exposure chamber using a proportional diluter (concentrated aqueous stock solutions will be prepared without using carrier solvents). The exposure is conducted for 21 days, during which time the appearance of the fish, behavior, and fecundity are assessed daily. Hatching success and post-hatch viability of larvae is monitored for 24 hours in control water. At termination of the exposure, blood samples are removed from adults and analyzed for sex steroids and VTG. The gonads are also removed for GSI determination and later histological analyses.

Description of the Method

Test animals and assay system

Test Animals: The assay will be started with newly mature fish (typically four to six months old), as opposed to older animals that have been actively reproducing for some period of time. Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable a fathead minnow culture will be maintained, as opposed to purchasing

the animals prior to testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 1.

Water: It is well established that the fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this assay. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 2. The animals will be tested using a flow-through water renewal system that maintains adequate water quality (temperature, dissolved oxygen, low ammonia, etc.), and ensures a consistent exposure to the parent chemical (for those tests where water is the route of exposure).

Assay System: Five-gallon glass exposure vessels are used for the assay system. As recommended by Ankley et al. (2001), dimensions of the assay chambers must be such that the animals can interact in a fashion conducive to successful spawning. The assay chamber contains 10 L of test solution, which is renewed at least once every 4 hours. This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality (Tables 4-1(1) and 4-2(2)), while not utilizing an excessive amount of test material.

Experimental Design

We are expecting that range-finding tests will not be necessary because guidance on test chemical concentrations is being provided by scientists from the U.S. EPA Mid-Continent Ecology Division and is shown in Section 3.0.

For the 21-day reproduction assay, the highest concentration of chemicals used should not have caused significant mortality in previous range-finding tests (note, this may be at water solubility), and the lower concentration/dose used should be a factor of 5 to 10 times lower than the highest test concentration. At present, a minimum of four replicate tanks (each containing four females and two males) is recommended per treatment. Based on this design, a minimum of 72 fish is required per assay (six fish in each of four replicates for two treatments, plus one control).

Exposure via Water

Water exposures will be conducted using a proportional diluter without a carrier solvent. When necessary, concentrated aqueous stock solutions of the test chemical will be prepared using a saturator method described by Kahl et al. (1999).

Analytical Determination

The concentrations of the test chemical in the exposure chambers will be measured prior to adding fish to verify that target concentrations are reached. Additionally, water samples will be removed weekly and analyzed for the test chemicals. All test chemicals will be directly measured using GC-MS (methyl-testosterone), GC-ECD (methoxychlor), and HPLC (Fadrozole and Flutamide). In some instances, concentration of the test chemical in aqueous stock

solutions will be estimated by UV-Vis spectroscopy to facilitate adjustment of the proportional diluter.

Performance of the Assay

Assay Initiation and Conduct

Pre-exposure: The 14-day pre-exposure phase of the assay will use five to six month-old minnows, previously maintained in communal culture tanks. Four females and two males should be randomly assigned to the replicate exposure chambers at each treatment concentration. Approximately 50% additional exposure chambers will be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure spawning. Any specimens whose gender cannot be identified will be excluded from the assay. It has been reported that, at 5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles, while females possess an ovipositor.

The pre-exposure phase of the assay is conducted under conditions (temperature, photoperiod, feeding, etc.) identical to those used during the chemical exposure (Table 2. The animals are fed frozen *Artemia* twice daily *ad libitum*. The fish will be monitored daily for alterations in secondary sex characteristics (breeding tubercles in males, ovipositor in females), reproductive behavior, and spawning activity. Hatching success of embryos will be evaluated twice during pre-exposure using 50 fertilized eggs. Gross appearance of newly hatched larvae also will be evaluated.

Chemical Exposure: After successful spawning during pre-exposure, the chemical exposure will be initiated and continued for 21 days. As reported by Ankley et al. (2001), this exposure duration should allow for collecting of sufficient data for assessments of fecundity and fertilization success, embryo development, and hatching success. In addition, the 21-day assay period should help optimize exposure of the fish to relatively hydrophobic chemicals, which require a period of time to reach steady-state concentrations in the animal.

Observations

A number of endpoints are assessed over the course of, and/or at conclusion of the 21-day assay. Below are described the collection of these endpoints and their utility, particularly in the context of the assay as an EDC screen.

Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/day.

Behavior of Adults: Abnormal behavior (relative to controls), such as hyperventilation, loss of equilibrium, and feeding abstinence, will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males also will be noted.

Fecundity: Egg production will be determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they will not be disturbed (except for feeding) until late morning. This allows time for spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates can be removed from the tanks to enumerate any eggs that are present. Based on the published report of this protocol, it is expected that one spawn typically will be composed of 50 to 150 eggs. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (test) day per replicate. Therefore, if all four females survived the treatment in a given replicate for the duration of 21 days, there would be 84 female reproductive days. Based on the results published by Ankley et al. (2001), the number of spawns is likely to decrease during the exposure phase of the assay as a result of the effects on reproduction caused by the test compounds.

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos are carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) is easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; Viable embryos remain clear for 36 to 48 hours until reaching the eyed stage.

Hatchability, Larvae Appearance, and Survival: At 25˚C, untreated animals will hatch in 4.5 to 6 days. Each incubation chamber should be evaluated daily for newly hatched embryos; this endpoint should be expressed as a relative percentage of those eggs deemed fertile. The hatching rate of control animals typically is in the range of 95 to 98%.

The appearance and behavior of hatched larvae can be evaluated and the results described either qualitatively or quantitatively (e.g., malformation rate). Gross morphological anomalies that may be observed include lordosis, scoliosis, kyphosis, retarded swim bladder development, and craniofacial abnormalities. Larvae survivability will be assessed until yolk sac absorption (ca. 96 hours at 25˚C).

Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.

Blood Sampling: At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L) and weighed. Blood will be collected from the caudal artery/vein with a heparinized

microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL. Plasma is separated from the blood via centrifugation (3 minutes at 15,000 x g) and stored with protease inhibitors at -80ºC until analyzed for VTG and sex steroids.

Gonad Size and Morphology

After sampling the blood, fish should be weighed and the gonads removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish will be discarded.

Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be placed in fixative (10% buffered formalin) and embedded in paraffin. Serial sections 4 to 5 µm thick will be cut along the long axis of the gonad. At least two serial sections will be collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six-tissue sections/sample. Sections will be stained with hematoxylin and eosin, and will be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. Evaluation of the testis is based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin

The measurement of VTG in plasma samples will be performed using an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, polyclonal carp (*Cyprinus carpio*) VTG antibody, and purified VTG protein also from the carp will be utilized.

Sex Steroids

Plasma concentrations of β-estradiol, testosterone, and 11-ketotestosterone will be determined using competitive enzyme immunoassays (EIAS) that are commercially available for each steroid of interest.

Performance Criteria

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 4-1(1) and 4-1(2).

- Documentation of purity of the test material must be provided by the vendor (for test compounds purchased commercially). Documentation will also be maintained for chemical analysis of exposure water (e.g., verification of exposure concentrations of the chemical in test water).

- There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every 3 to 4 days.

- There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

Data Reporting

Treatment and interpretation of results

Any endpoints that are significantly impacted by the test chemical will be reported as such.

The test report will include the following:

Test substance:

- The report will include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.

- Additional information should be provided, when available, such as its solubility in water, octanol : water partition coefficient, vapor pressure, and toxicity to fathead minnow.

Test species:

- Information must be provided on the fathead minnows used in the assay. This information must include the source of the fish, age and condition of the fish at the initiation of the assay, and the pre-exposure reproductive performance.

- Any observed abnormalities in reproductive behavior or performance of control fish also must be reported.

Test conditions: The report must specify the conditions under which the assay was performed, this includes

- Information on the source, treatment of, and basic chemical characteristics of the dilution water
- Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity, and conductivity
- The photoperiod and light intensity used during the exposure
- The chamber size, number of females and males per replicate, and number and composition of spawning substrates
- Information on food provided during the exposure, including supplier and lot number.
- Detailed information on the flow-through water delivery system, specifically, daily number of volume exchanges of dilution water.

Results:

- The results must include data for the control and the treatment fish.

- The table of results must include the mean, standard deviation and range for each assay endpoint from the replicates employed in the assay. Statistical significance of means should be indicated.

4.2 Abbreviated Short-term (14-day) Reproduction Assay with the Fathead Minnow for Identification of Potential Endocrine Disrupting Chemicals

Introduction

This protocol describes a 14-day reproduction assay using the fathead minnow *(Pimephales promelas)*. This assay is an abbreviated version of the 21-day assay described previously and is intended as a less intensive alternative. As with the 21-day assay, the 14-day abbreviated assay measures the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints are performed to aid identification of the specific toxicological mode of action of the test chemical.

The assay is started with mature male and female fish previously observed for spawning activity. During the subsequent 14-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed; and daily fecundity and fertilization success measurements are recorded. Successful embryological development is measured based on the hatching success of fertilized eggs. At termination of the assay, measurements are made of external morphology, GSI, gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT).

Principle of the Assay

An overview of the assay and relevant assay conditions is provided in Table 4-2(1). The assay is initiated with mature adults that appear to be reproductively active as determined by observations of spawning that are made during the 14-day pre-exposure phase. No quantitative measures of fecundity or embryo viability (e.g., hatchability) are made during the pre-exposure phase. As with the 21-day assay, the 14-day assay is conducted using two chemical concentrations, as well as a control with four experimental units (replicates) per treatment. Each replicate tank contains four female and two male fish. The test chemical is delivered to the exposure chamber using a proportional diluter (concentrated aqueous stock solutions will be prepared without using carrier solvents). The exposure is conducted for 14 days, during which time the appearance of the fish, behavior, and fecundity are assessed daily. Hatching success and post-hatch viability of larvae is monitored for 24 hours in control water. At termination of the exposure, blood samples are removed from adults and analyzed for sex steroids and VTG. The gonads are also removed for GSI determination and later histological analyses.

Description of the Method

Test Animals and Assay System

Test Animals: The assay will be started with newly mature fish (typically four to six months old), as opposed to older animals that have been actively reproducing for some period of time. To maintain a ready supply of known-quality animals at the desired age for routine testing, a fathead minnow culture will be maintained, as opposed to purchasing the animals prior to testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 1.

Water: It is well established that the fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this assay. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 4-2(2). The animals will be tested using a flow-through water renewal system that maintains adequate water quality (temperature, DO, low ammonia, etc.), and ensures a consistent exposure to the parent chemical (for those tests where water is the route of exposure).

Assay System: Five-gallon glass exposure vessels are used for the assay system. As recommended by Ankley et al. (2001), the dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. The test chamber contains 10 L of test solution, which is renewed at least once every 4 hours. This particular animal loading/water renewal rate is within recommended guidelines and, in studies conducted according to this method, has maintained acceptable water quality (Tables 4-2(1) and 4-2(2), while not utilizing an excessive amount of test material.

Experimental Design

We are expecting that range-finding tests will not be necessary because guidance on test chemical concentrations is being provided by scientists from the U.S. EPA Mid-Continent Ecology Division.

For the 14-day reproduction assay, the highest concentration of chemicals used should not have caused significant mortality in previous range-finding tests (note, this may be at water solubility), and the lower concentration/dose used should be a factor of 5 to10 times lower than the highest test concentration. Four replicate tanks (each containing four females and two males) will be per treatment. Based on this design, a minimum of 72 fish is required per assay (six fish in each of four replicates for two treatments, plus one control).

Exposure via Water

Water exposures will be conducted using a proportional diluter without a carrier solvent. When necessary, concentrated aqueous stock solutions of the test chemical will be prepared using a saturator method described by Kahl et al. (1999).

Analytical Determination

The concentrations of the test chemical in the exposure chambers will be measured prior to adding of fish to verify that target concentrations are reached. Additionally, water samples will be removed weekly and analyzed for the test chemicals. All test chemicals will be directly measured using GC-MS (methyl-testosterone), GC-ECD (methoxychlor), and HPLC (Fadrozole and Flutamide). In some instances, the concentration of the test chemical in aqueous stock solutions will be estimated be by UV-Vis spectroscopy to facilitate adjustment of the proportional diluter.

Performance of the Assay

Assay Initiation and Conduct

Pre-exposure: The 14-day pre-exposure phase of the assay will use five to six months-old minnows, previously maintained in communal culture tanks. Four females and two males should be randomly assigned to the replicate exposure chambers at each treatment concentration. Approximately 50% additional exposure chambers will be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure spawning. Any specimens whose gender cannot be identified will be excluded from the assay. It has been reported that at 5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles while, females possess an ovipositor.

The pre-exposure phase of the assay is conducted under conditions (temperature, photoperiod, feeding, etc.) identical to those used during the chemical exposure (Table 2). The animals are fed frozen *Artemia* twice daily ad libitum. The fish will be observed daily for spawning activity.

Chemical Exposure: After observing that spawning is occurring during the 14-day pre-exposure period, the chemical exposure will be initiated and continued for 14 days. Data will be collected for fecundity, fertilization success, embryo development, and hatching success.

Observations

A number of endpoints are assessed over the course of, and/or at the conclusion of, the 14-day assay. Below is described the collection of these endpoints.

Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/day.

Behavior of Adults: Abnormal behavior (relative to controls) such as hyperventilation, loss of equilibrium, and feeding abstinence, will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males also will be noted.

Fecundity: Egg production will be determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they will not be disturbed (except for feeding) until late morning. This allows time for spawning and fertilization to be completed, and for eggs to water-harden. The spawning substrates can be removed from the tanks to enumerate any eggs that are present. Based on the published report of this protocol, it is expected that one spawn My be composed of as many as 50 to 150 eggs. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (assay) day per replicate. Therefore, if all four females survived the treatment in a given replicate for the duration of 14 days, there would be 56 female reproductive days.

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos are carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) is easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated, Viable embryos remain clear for 36 to 48 h until reaching the eyed stage.

Hatchability, Larvae Appearance, and Survival: This endpoint will be assessed on two separate days during the exposure. At 25˚C, untreated animals will hatch in 4.5 to 6 days. Each incubation chamber should be evaluated daily for newly hatched embryos; this endpoint should be expressed as a relative percentage of those eggs deemed fertile. The hatching rate of control animals typically is in the range of 95 to 98%.

The appearance and behavior of hatched larvae can be evaluated and the results described either qualitatively or quantitatively (e.g., malformation rate). Gross morphological anomalies that may be observed include lordosis, scoliosis, kyphosis, retarded swim bladder development, and craniofacial abnormalities. Larvae survivability will be assessed until yolk sac absorption (ca. 96 hours at 25˚C).

Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations and any unusual changes will be noted. External features of particular importance include: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.

Blood Sampling: At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg NaHCO3/L), and weighed. Blood will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL. Plasma is separated from the blood via centrifugation (3 minutes at 15,000 x g) and stored with protease inhibitors at -80ºC, until analyzed for VTG and sex steroids.

Gonad Size and Morphology

After sampling the blood, fish should be weighed and the gonads removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish will be discarded.

Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be placed in fixative (10% buffered formalin) and embedded in paraffin. Serial sections 4 to 5 μm thick will be cut along the long axis of the gonad. At least two serial sections will be collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six-tissue sections/sample. Sections will be stained with hematoxylin and eosin, and will be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. Evaluation of the testis is based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin

The measurement of VTG in plasma samples will be performed using an ELISA. For the ELISA, polyclonal Carp (*Cyprinus carpio*) VTG antibody, and purified VTG protein also from the carp will be utilized.

Sex Steroids

Plasma concentrations of β-estradiol, testosterone, and 11-ketotestosterone will be determined using competitive EIA that are commercially available for each steroid of interest.

Performance Criteria

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2.
- Documentation of purity of the test material must be provided by the vendor (for test compounds purchased commercially). Documentation will also be maintained for chemical analysis of exposure water (e.g., verification of exposure concentrations of the chemical in test water).
- There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every 3 to 4 days.
- There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

Treatment and interpretation of results

Any endpoints that are significantly impacted by the test chemical will be reported as such.

Test report

The test report will include the following:

Test substance:

- The report will include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.
- Additional information should be provided, when available, such as its solubility in water, octanol : water partition coefficient, vapor pressure, and toxicity to fathead minnow.

Test species:

- Information must be provided on the fathead minnows used in the assay. This information must include the source of the fish, age and condition of the fish at the initiation of the assay, and the pre-exposure reproductive performance.
- Any observed abnormalities in reproductive behavior or performance of control fish also must be reported.

Test conditions: The report must specify the conditions under which the assay was performed, this includes

- Information on the source, treatment of, and basic chemical characteristics of the dilution water
- Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity and conductivity
- The photoperiod and light intensity used during the exposure
- The chamber size, number of females and males per replicate, and number and composition of spawning substrates
- Information on food used to feed the fish during the exposure, including supplier and lot number
- The basic nature of exposure (i.e. flow-through, ip injection or dietary) in addition to specific information related to the exposure type (e.g. whether flow-through water delivery type, daily number of volume exchanges of dilution water)
- Use of solvent or dispersant if any, the specific solvent or dispersant and the concentrations to which the fish were exposed must be specified.

Results:

- The results must include data for the control and the treatment fish.
- The table of results must include the mean, standard deviation, and range for each assay endpoint from the replicates employed in the assay. Statistical significance of means should be indicated.

4.3 14-day Fish Endocrine Screening Assay (14 day OECD assay)

Introduction

This assay protocol describes a 14-day bioassay for identifying EDCs in adult fathead minnows based on the protocol described by the OECD Draft proposal–31 December 2001. The assay is intended to detect the EDCs with a mode of action that is (anti)-androgenic or (anti)-estrogenic. This 14-day assay is intended to identify changes in biochemical, morphological, and histological endpoints considered to be endocrine specific in fathead minnows. Reproductive performance parameters are not assessed in this test protocol. The concept for this protocol is derived from work in Europe on the fathead minnow (*Pimephales promelas*) (Panter et al., 1998a and 1998b) and in North America (Ankley et al., 2001).

This test guideline addresses a 14-day chemical exposure period that does not require a pre-exposure validation period. The assay is started with mature male and female fish. During the subsequent 14-day chemical exposure, survival and secondary sexual characteristics are observed. At termination of the assay, measurements are made of external morphology, gonadosomatic index (GSI), gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT). This 14-day period may be reduced if the exposed fish exhibit a clear response to the test chemical in terms of altered secondary sexual characteristics [most likely to occur during methyl-testosterone exposures (Ankley et al, 2001)].

Principle of the Assay

An overview of the assay and relevant test conditions is provided in Table 3. The assay is initiated with healthy, sexually dimorphic adult fish (males and females contained in separate test chambers to prevent induction of spawning). To minimize the confounding effect of natural spawning cycles, these fish are selected from pre-spawning populations. The assay will be conducted using three chemical concentrations, as well as a controls, with two individual vessels per treatment (one vessel containing 10 males, the other 10 females). Chemical delivery is via a flow-through water system using a proportional diluter. The assay duration is 14 days, during which the behavior and appearance of the fish are observed daily. At the end of the assay fish are terminated in oxygenated MS-222 solution and blood samples are collected for determination of vitellogenin and sex steroids as described for the 14/21 day reproductive performance assays. The gonads are removed for measurement of the GSI and histological analyses. Early signs of a treatment induced effect, such as male fish appearing feminized, may indicate that completing other endpoint analyses (vitellogenin and gonad histology) is not necessary. In all cases, however, blood and gonads must be collected to support the gross observations with biochemical (vitellogenin) or histological endpoints, if necessary.

Description of the Method

Test Animals and Assay System

Test animals: The assay should be started with mature fish (at least five to six months old). Test fish will be selected from a population of a single stock, preferably from the same spawning, acclimated for at least two weeks prior to the assay under conditions of water quality and illumination similar to those used in the assay. Fish will be fed a ration of 1% body weight per day throughout the holding period and during the 14-day bioassay.

Water: No specific water type is required for this assay. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 2. The animals will be tested using a flow-through water renewal system that maintains adequate water quality (temperature, DO, low ammonia, etc.), and ensures a consistent exposure to the parentchemical (for those assays where water is the route of exposure).

Assay system: Five-gallon glass exposure vessels will be used for the test system. As recommended by Ankley et al. (2001), the dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. Each test chamber contains 10 L of test solution, which is renewed at least once every four hours. This particular animal loading/water renewal rate is within recommended guidelines and has maintained acceptable water quality in studies conducted according to this method (Tables 1 and 2), while not using an excessive amount of test material.

Experimental Design

Range-finding tests are expected to be unnecessary because of guidance on test chemical concentrations is provided by scientists from the U.S. EPA Mid-Continent Ecology Division.

For the 14-day assay, the highest concentration should not have caused significant mortality in previous range-finding tests (note, this may be at water solubility) and the lower concentration/dose should be a factor of 5 to 10 times lower than the highest test concentration. The third exposure concentration will be intermediate between the high and low concentrations in the 14/21-day reproductive performance assays. Two exposure tanks are used for each test concentration. One replicate will contain 10 males and the other replicate will contain 10 females. Two control tanks also are used containing 10 males and 10 females. Based on this design, a minimum of 80 fish is required per assay (20 fish in each of three treatments, plus one control).

Exposure via Water

Water exposures will be conducted using a proportional diluter without a carrier solvent. When necessary, concentrated aqueous stock solutions of the test chemical will be prepared using a saturator method described by Kahl et al. (1999).

Analytical Determination

Water concentrations of the test chemical in the exposure chambers will be measured prior to adding fish to verify target concentrations. Additionally, water samples will be analyzed weekly for the test chemicals. All test chemicals will be directly measured using GC-MS (methyl-testosterone), GC-ECD (methoxychlor) and HPLC (Fadrozole and Flutamide). In some instances, the concentration of the test chemical in aqueous stock solutions will be estimated by UV-Vis spectroscopy to facilitate adjustment of the proportional diluter.

Performance of the Assay

Assay Initiation and Conduct

Chemical exposure: After the acclimatization period, spawning chemical exposure will be initiated and continued for 14 days during which time behavior and appearance of the fish will be observed on a daily basis.

Observations

Fish will be examined daily during the assay period, and any external abnormalities (such as hemorrhage or discoloration) will be noted. Any mortality will be recorded and the dead fish removed as soon as possible. Dead fish will not be replaced.

Behavior of Adults: Abnormal behavior (relative to controls) such as hyperventilation, loss of equilibrium, or feeding abstinence will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, also will be noted.

Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.

Termination at 14-days: Gonad size and morphology, and biochemical endpoints (VTG, Steroids)

Blood collection: At conclusion of the exposure, fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L) and weighed, and blood will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon fathead minnow size (which usually is sex dependent), blood volumes generally range from 30 to 80 µL. Plasma is separated from the blood via centrifugation

(3 min at 15,000 x g) and stored with protease inhibitors at -80ºC until analyzed for VTG and sex steroids.

Gonad size and morphology: After blood sampling, fish should be weighed, and the gonads will be removed and weighed (to the nearest 0.1 mg) for determination of the GSI (GSI = 100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the fish carcass will be discarded.

Routine histological procedures will be used to assess the condition of the testes and ovaries from the fish. Gonads will be placed in fixative, (10% buffered formalin) and embedded in paraffin. Serial sections 4 μm to 5 μm thick will be cut along the long axis of the gonad. At least two serial sections will be collected from at least three areas equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample. Sections will be stained with hematoxylin and eosin and will be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. Evaluation of the testis is based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin: VTG in plasma samples will be measured using an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, polyclonal Carp (*Cyprinus carpio*) VTG antibody and purified VTG protein also from the carp will be used.

Sex steroids: Plasma concentrations of β-estradiol, testosterone, and 11-ketotestosterone will be determined using competitive enzyme immunoassays (EIA) that are commercially available for each steroid of interest.

Performance Criteria

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2.
- Purity of the test material must be documented by the vendor (for test compounds purchased commercially). Documentation also will be maintained for chemical analysis of exposure water (e.g., verification of exposure concentrations of the chemical in test water).

Data Reporting

Treatment and interpretation of results

Any endpoints that are significantly impacted by the test chemical will be reported as such.

Test report

The test report will include the following:

Test substance:

- The report will include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.
- Additional information should be provided, when available, such as solubility in water, octanol:water partition coefficient, vapor pressure, and toxicity to fathead minnow.

Test species:

- Information that must be provided on the fathead minnows used in the assay includes the source of the fish, age and condition of the fish at the initiation of the assay, and the pre-exposure reproductive performance.
- Any observed abnormalities in reproductive behavior or performance of control fish must also be reported.

Assay conditions: The report must specify the following conditions under which the assay was performed:

- Source, treatment, and basic chemical characteristics of the dilution water
- Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity, and conductivity
- Photoperiod and light intensity used during the exposure
- Chamber size, number of females and males per replicate, and number and composition of spawning substrates
- Food used to feed the fish during the exposure, including supplier and lot number
- Basic nature of exposure (i.e. flow-through) in addition to specific information related to the exposure type (e.g. the number of volume exchanges of dilution water daily)
- Use of solvent or dispersant if any, the specific solvent or dispersant and the concentrations to which the fish were exposed.

Results:

- The results must include data for both control and treated fish.
- The table of results must include the mean, standard deviation, and range for each assay endpoint from the replicates employed in the assay. Statistical significance of means should be indicated.

Table 1. Test Conditions for the 14 Day or 21 Day Fathead Minnow EDC Screening Assay with Measurement of Reproductive Performance

Test type	
Water temperature	25 ±1°C
Illumination	Quality fluorescent bulbs (wide spectrum)
Light intensity	10-20 µE/M2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
Photoperiod	16 h light, 8 h dark
Test chamber size	18 L (40 x 20 x 20 cm) (minimum)
Test solution volume	10 L
Volume exchanges of test solutions	Minimum of six daily
Flow rate	Approx 3.5 L/g fish/day
Age of test organisms	Reproducing adults (120-day minimum)
No. of fish per test chamber	4 females and 2 males
No. of treatments	2 (plus appropriate controls)
No. of replicate test chambers per treatment	4
No. of fish per test concentration	16 females and 8 males
Feeding regime	Frozen adult brine shrimp twice daily
Aeration	None unless DO concentration falls below 4.9 mg/L
Dilution water	Clean surface, well, or reconstituted water
Dilution factor	5-10
Chemical exposure duration	£21-d (21-day test) £14-d (14-day test)
Endpoints	Adult survival, reproductive behavior, secondary sexual characteristics, GSI and gonadal histology, plasma VTG and sex steroids (b-estradiol, testosterone, 11-KT) concentrations, fecundity, fertility, embryo hatch, and larval survival
Test acceptability	Dissolved oxygen >60% of saturation; mean temperature of 25 ±1°C, 90% survival in the controls, successful egg production in controls

Table 2. Recommended Ranges of Water-quality Characteristics for Culturing and Testing the Fathead Minnow

Water characteristic	Preferred range	
Temperature (°C)	24.0-26.0	Alcohol or electronic thermometer
Dissolved oxygen (mg/L)	>4.9 mg/L (≥60% saturation)	Iodometric or membrane electrode
pH	6.5-9.0	Electronic meter
Total alkalinity (mg/L as $CaCO_3$)	>20	Acid titration
Total organic carbon (mg/L)	≤5	TOC analyzer
Unionized ammonia (µg/L)	<35	Nesslerization with pH and temperature adjustments

Table 3. Test Conditions for the OECD 14-Day Fish Endocrine Screening Assay Guideline

Test type	
Water temperature	25 ± 2ºC
Illumination	Fluorescent bulbs (wide spectrum)
Light intensity	10-20 µE/M2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
Photoperiod	16 h light, 8 h dark
Test chamber size	18 L (40 x 20 x 20 cm) (minimum)
Test solution volume Volume exchanges of test solutions	10 L Minimum of five daily
Flow rate	Approx 3.5 L/g fish/day
Age of test organisms	Pre-breeding Adults (5-6 months)
Weight of fish	Females: 1.5 +/- 10%; Males 2.5 +/- 10%
No. of fish per test chamber	10
No. of treatments	3 minimum (plus appropriate controls)
No of test chambers per treatment	2
No. of fish per test concentration	10 females and 10 males
Feeding regime	Frozen adult brine shrimp twice daily
Aeration	None unless DO concentration falls below 4.9 mg/L
Dilution water	Clean surface, well, or reconstituted water
Dilution factor	3-10
Pre-exposure period	None
Chemical exposure duration	≤14-d
Endpoints	Adult survival and behavior, secondary sexual characteristics, GSI and gonadal histology, plasma VTG and sex steroids (b-estradiol, testosterone, 11-KT) concentrations
Test acceptability	Dissolved oxygen >60% of saturation, mean temperature of 25 ± 2ºC, 90% survival in the controls

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