DRAFT PROPOSAL FOR A NEW GUIDELINE

Fish Two-generation Test Guideline

INTRODUCTION

1. This guideline describes a two-generation test with fish that considers reproductive fitness in parents and offspring as an integrated measure of toxicant. It also enables measurement of a suite of histological and biochemical endpoints that allow diagnostic and definitive evaluation of endocrine disrupting chemicals (EDCs) or other types of reproductive toxicant. This guideline is intended to be applicable to the fathead minnow *(Pimephales promelas),* medaka (*Oryzias latipes*), sheepshead minnow (*Cyprinodon variegatus*) and zebrafish (*Danio rerio*). The fish two-generation test is a relatively long-term (normally 180-d or longer) assay that assesses early development, growth, and reproduction. The two-generation test described in this guideline combines elements of the recently developed short-term reproduction test (Ankley *et al.,* 2001) and life-cycle toxicity test methods developed for fathead minnow (Benoit, 1982) and sheepshead minnow (Hansen *et al*., 1978).

2. The test is intended to serve as a higher tier for collecting definitive concentration-response information on adverse effects suitable for use in ecological risk assessment. Specifically, the design enables the collection of both mechanism-specific toxicity data and information concerning fecundity and viability. The former is useful as a basis for diagnosis and/or extrapolation across species, while the latter is critical to application of population models to risk assessment scenarios.

3. The two-generation test is initiated with mature male and female (P generation) fish, eggs are collected and F1 embryo fertility, development, sexual maturation, reproduction and F2 viability are also assessed. Measurements are made of a number of endpoints in both P and F1 generations reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology and plasma or whole body concentrations of vitellogenin. Additionally, plasma sex steroids (17β-estradiol, testosterone, 11-ketotestosterone) and thyroid hormones (T3/T4) may also be measured if it is considered that additional useful information may be generated (Ankley *et al*., 2001).

PRINCIPLE OF THE TEST

4. An overview of the methodology and relevant test conditions is provided in Table 1 (Annex 1). Parental fish exposure (P) - For the recommended species (namely, fathead minnow, medaka, sheepshead minnow, and zebrafish), the test protocol 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 7 to 21-d pre-exposure period in the same test system as will be utilized for the chemical exposure phase. The test is conducted at a minimum of five chemical concentrations, as well as appropriate controls, with a minimum of four (three replicates for the zebrafish) experimental units (replicates) per treatment. In general, each replicate tank contains four female and two male fish, however, for zebrafish each replicate tank should contain at least 6 individuals (proportionally distributed 1 female to 2 males). The

exposure for P fish is conducted for 21-d, during which appearance of the fish, behavior, and fecundity are assessed daily.

5. Exposure of F1 generation - Viability of resultant embryos (e.g., hatching success, developmental rate, occurrence of malformations, etc.) is assessed in animals held in the same treatment regime to which the adults were exposed. Between fifty (50) and one hundred (100) embryos produced on day 21 (or as soon thereafter) are transferred to brooding chambers for each replicate under the same treatment regime to which the adults were exposed. After hatching, juvenile F1 fish are reduced to a minimum number of 25 and a maximum of 50 per brooding chamber. For species with secondary sex characteristics, F1 fish are thinned to 4 females and 2 males per replicate spawning chamber. For species where sex is difficult to determine by external characterixtics (*e.g*., zebrafish), F1 fish are thinned to at least 6 individuals per brooding chamber. Spawning and embryo viability are assessed daily until 21 days beyond the mean initial spawn date of controls. At conclusion of the test, blood samples or whole body are collected from the adults (F1) for determination of sex steroids and vitellogenin, and the gonads sampled for measurement of the GSI and histological analyses. Genetic sex ratio and survival of F2 juveniles is assessed.

6. Exposure of F2 generation – Viability of resultant embryos from the F1 (*i.e*., hatching success, developmental rate, occurrence of malformations, etc.) is assessed in animals held in the same treatment regime to which the adults were exposed. At termination of the test (at sexual maturation of the F2 generation), samples are collected for analysis of sex steroids and vitellogenin. Gonads are sampled for measurement of the GSI, histological analyses, and phenotypic sex ratios. If possible, genetic sex ratio should be confirmed.

DESCRIPTION OF THE METHOD

Test animals and assay system

Test animals

7. The test should be started with newly mature fish as opposed to older animals that have been actively reproducing for some period of time, for example, in a brood stock production facility. Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable to maintain a fish culture, as opposed to purchasing the animals prior to testing. Field-collected fish generally should not be used to initiate cultures or for testing. Information on general culture and testing requirements are provided in the literature for most OECD fish species, including the fathead minnow (USEPA 1982 & 1987; ASTM 1993), medaka (Gray and Metcalfe, 1999; Groonen *et al*, 1999; Shioda and Wakabayashi, 2000), sheepshead minnow (Hansen and Parrish 1977; Hansen et al. 1978; and Schimmel and Hansen 1974) and zebrafish (Bresch *et al*, 1990; Andersen *et al*, 2000; Andersen *et al*, 2002; Van den Belt *et al*, 2001).

Water

8. Fathead minnows, medaka, and zebrafish can reproduce successfully over a wide range of freshwater quality. Therefore, no specific freshwater type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended freshwater quality characteristics are listed in Table 2 (Annex 2). Sheepshead minnows require saltwater and any uncontaminated natural filtered or reconstituted seawater adjusted to 25 $\pm$ 3 ppt salinity

should be acceptable. The animals should be tested using a flow-through or automated water renewal system that enables maintenance of adequate water quality (temperature, dissolved oxygen, low ammonia, salinity, etc.), as well as ensuring a consistent exposure to the test chemical (for those tests where water is the route of exposure). There is no absolute requirement for the physical apparatus used to supply water and test chemical, except that all materials be chemically inert. Water needs to be delivered at a controlled rate to maintain consistent water quality and chemical exposure, but several options are available. For example, the water/chemical delivery apparatus for the test may be modified from gravity-feed proportional diluter systems, of which several designs are available, or could be based on systems that utilize pumps.

Assay system

9. The design and materials used for the exposure system are optional; basically, any system that enables conformance with the performance-based criteria described below, related to chemical delivery, water quality, and animal health is acceptable. Glass, stainless steel, or other chemically inert material should be used for construction of the test system. The dimensions of the test chambers must be such that the animals can interact in a fashion conducive with successful spawning. For the recommended species (namely, fathead minnow, medaka, sheepshead minnow, and zebrafish), successful fish breeding has been achieved using glass chambers of dimensions 40(l) x 20(w) x 25(h) cm containing three spawning substrates made from suitably inert material (Makynen et al 2000; Ankley et al 2001) for substrate spawners (e.g., fathead minnow) or three spawning areas with removable trays covered with mesh screen (Hansen et al., 1978) for broadcast spawners (e.g., sheepshead minnow) or other suitable test chambers. The test chamber contains 10 L of test solution, which should be renewed by flow-through at least 1 to 6 times daily.

10. Embryo incubation cups may be constructed from glass jars or cylinders or an appropriate size to contain the embryos. The bottoms of the jars should be cut off and replaced with stainless steel or nylon screen of a mesh size sufficient to retain newly-fertilized eggs but not so small as to become easily clogged (e.g., 16 mesh per cm).

Range finding test

11. Preliminary testing is recommended to determine the appropriate concentrations to use in the definitive life cycle toxicity test. The form of preliminary testing will depend upon existing toxicological information available on the test material. Ideally, the results of a 96-hour toxicity test with juvenile fish (e.g., OECD TG 203), an early life stage toxicity test (e.g., OECD TG 210) and/or a short term reproduction toxicity assay (e.g., Ankley et al. 2001) would be available. If such data are not available then a range-finding test should be conducted under conditions (water quality, test system, animal loading) similar to those intended for the reproduction test (see Table 1). It should utilize juvenile fish, and focus on lethality over the course of at least a 4- to 7-d assay. The maximum concentration to be used in a range finding series should be at the limit of water solubility or up to a maximum nominal test concentration of 100 mg per L, as per standard OECD practice; in general, a logarithmic dilution series should suffice for the range-finding assay.

Definitive test

Experimental design

Test concentrations

12. Normally, five concentrations are used. The highest concentration used is selected from existing information or preliminary testing (range finding test) to not cause significant acute mortality, but be expected to adversely affect a sublethal endpoint. Subsequent concentrations/doses used should be stepped down by at least a factor of 2. The lowest concentration must not affect any life stage. The use of five test concentrations in this fashion not only enables at least some consideration of unexpected dose-response relationships, but should provide for determination of a No Observed Effect Concentration (NOEC) or an ECx .

Controls

13. Every test includes controls consisting of the same dilution water, conditions, procedures, and test population, except no test material is added. Carrier (solvent) controls are required if a solvent is used.

Replicates and number of test organisms

14. A minimum of four replicate tanks per treatment are required (three for the zebrafish). Spawning adults in both the parental (P) and first filial (F1) phases should be allocated into groups of four females and two males per replicate tank for fathead minnow, medaka, and sheepshead minnow. For zebrafish, where sexes are not obviously discernible, an aggregate of $\geq$ 6 adults are used. Between 50 and 100 embryos from the first filial (F1) terminal spawn and second filial (F2) terminal spawn are allocated per replicate. In the F1 post-hatch/growth phase, 25 to 50 juveniles are allocated within each replicate from those hatched.

Administration of test substance and analytical determination

15. Theoretically there are several options for delivery of test chemical to the fish: via the water, intraperitoneal (ip) injection or diet.

Exposure via water

16. From a practical perspective, however, only variations on the aqueous route have been used with any frequency in testing with the recommended species (namely, fathead minnow, medaka, sheepshead minnow, and zebrafish). Water exposures can be conducted either with or without a solvent carrier for the test chemical of concern. When a chemical is relatively soluble in water (ionic compounds) a solvent is not required to enhance water solubility for preparation of stock solutions; however, much of the toxicity testing historically conducted with aquatic animals and sparingly soluble nonionic chemicals has utilized carrier solvents. This can be problematic in numerous respects; therefore, it is recommended that, whenever possible, solvents not be used when generating stock solutions for conducting chemical exposures via the water.

17. The use of liquid-liquid and solid-liquid saturators to generate solvent-free stock solutions for aqueous testing has become common in recent years, and methods have been described using these approaches to test chemicals with widely varying physicochemical properties, including some considered to be very insoluble in water (Makynen et al, 2000).

18. On occasion it may be necessary to utilize solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. In general, the toxicity of common solvents has not been fully evaluated in chronic tests and there are relatively few studies on the possible effects of these solvents on

the fish endocrine system. Hence, it is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed controls.

Other routes of exposure

19. Since most chronic fish tests focus on water as the most important environmentally relevant route of exposure, relatively little work has been done exposing the recommended test species chemicals via intraperitoneal injection or the diet. These routes might be considered for many of the same reasons that would result in the use of carrier solvents in aqueous exposures, that is, insolubility, instability, and/or limited availability of the test chemical.

Analytical determination

20. Regardless of the exposure technique utilized for this assay, supporting analytical chemistry is critical to (i) ensure chemical purity, (ii) document that the test chemical is reaching the fish, and (iii) confirm system performance. In the water exposures, concentrations of the (parent) chemical should be measured at the start of the assay, at least weekly in the stock solution(s) and in each of the test treatments. When using the intraperitoneal or dietary routes, concentrations of the chemical in the injection media and food, respectively, should be measured. Regardless of the route of exposure utilized, at conclusion of the assay it may be useful to measure residues of the parent chemical and, if relevant, active metabolites in fish tissues.

PERFORMANCE OF THE TEST

Test initiation and conduct

Pre-exposure

21. The pre-exposure phase of the test should be started with animals that have achieved reproductive maturity, as evidenced by initial development of secondary sex characteristics (except in zebrafish where age would criteria), but have not been held in a culture/test situation conducive to routine spawning. These animals, which typically are 3 to 4 months old, are held in a "mass" culture tank that corresponds to the date the animals were hatched. They typically represent the pooled offspring of 10 to 20 pairs of adult fish from the culture facility. Four females and two males (or optimal number of spawning pairs for zebrafish) should be randomly assigned to the replicate exposure chambers at each anticipated treatment concentration. In addition to the number of replicates actually exposed at each concentration (e.g. three or four), a number of additional tanks also should be started; these can serve as "replacement" units for tanks in which pre-exposure spawning is not observed, and/or mortality occurs before initiation of the chemical exposure. Identification of gender may be difficult to resolve for some individual fish; these animals should not be used for the assay. For zebrafish, where gender is never obvious, spawning groups should be in the sex ratio proportion of 1 female and 2 males in the group (normally at least 6 randomly selected individuals would provide > 98% confidence).

22. The pre-exposure phase of the test is conducted under conditions (temperature, photoperiod, feeding, etc.) identical to those used during the chemical exposure (Table 2). The animals are fed frozen brine shrimp (*Artemia* sp.) twice daily *ad libitum*. The fish should be monitored daily for obvious alterations in secondary sex characteristics (breeding tubercles in males, ovipositor in females), reproductive behavior, and spawning activity. If practicable, it may be desirable to assess aspects of development of resultant embryos, such as

hatching success and rate, and gross appearance of newly hatched larvae. This pre-exposure monitoring phase establishes both reproductive capacity of the test animals, and provides tank-specific baseline data for potential statistical comparison after initiation of chemical exposure.

23. The pre-exposure phase of the test should be between 7 and 21-d. If acceptable spawning has not occurred within 21-d, an assessment should be made as to why satisfactory biological performance had not been achieved. This might entail examination of water quality or condition of the fish. Minimal criteria for acceptable pre-exposure performance would include: (i) 100% survival of all adults, (ii) presence of eggs in each replicate tank once a week, and (iii) >80% fertilization of the eggs.

Chemical exposure

24. When successful spawning has been established, the chemical exposure can be initiated. The spawning groups are transferred from pre-exposure tanks to separate exposure tanks. The exposure portion of the test should not be initiated until all aspects of the delivery system are confirmed to have been functioning properly for 48 hours. This includes verification of stable test material concentrations and environmental conditions such as temperature, dissolved oxygen, and lightning. The target exposure duration for this first parental cohort (P) is 21-d, which is sufficient for healthy females to produce several clutches of eggs. This allows for a robust data set for assessments of sexual development (e.g., associated with egg maturation), fecundity and fertilization success.

25. On day 21 or as soon after successful spawning, 50 to 100 embryos are transferred to the incubation chambers and held under the same treatment regime to which the parents were exposed. Embryos are observed at least once daily until hatching is complete. Dead embryos will usually turn opaque and must be counted and removed each day. Live fungused embryos must also be counted and removed daily. If no concentration-effect relationship exists for the presence of fungus, the number affected should be subtracted from the original total when calculating percent hatch.

26. When hatching begins, the number hatched each day is recorded either by direct counts or by counting the number of embryos present and subtracting that number from the total number of embryos when hatching began.

27. When hatching is 90% complete, the total number of live larvae are counted, reduced to between 25 to 50 per incubation chamber and then released into the growth chamber. The number of live normal larvae and the number of deformed live larvae at each test treatment is recorded. Time to complete hatch (90%) in each chamber is recorded to the nearest day.

28. The larvae are fed newly hatch brine shrimp (*Artemia* sp.) nauplii. Observations for survival and abnormal effects are performed at least once a week. Larvae which die during the first two weeks after hatching deteriorate rapidly and are easily overlooked. Therefore, survival during this period is determined by counting the number of live fish.

29. Total length of the fish is measured at four and eight weeks following hatching. This may be done by draining the water to a minimal depth (1-2 com), placing the test chamber on a photographic light table with a translucent millimeter grid overlay, and taking photographic prints which may then be enlarged to facilitate measurements (McKim and Benoit 1971).

30. Secondary sexual characteristics may appear in 12-16 weeks for all species except the zebrafish. Once sex can be determined for fathead minnow, sheepshead minnow, and medaka, F1 fish are thinned to 4 females and 2 males per replicate spawning chamber. For zebrafish, F1 fish are thinned at 12 weeks post-hatch to $\geq$ 6 fish per replicate spawning chamber. Observation for sexual development and spawning activity should continue. All surplus fish are weighed and measured, positively sexed, and sampled for biochemical measures and gonad histology.

31. Spawning substrates are checked daily for embryos. All embryos produced in each spawning are counted and removed. Fifty (50) to one hundred (100) embryos (F2) are impartially selected from each treatment 21 days after spawning first begins in controls and transferred to incubation chambers, as previously described for F1 embryos. Remaining embryos are discarded. Exposure of the adult F1 fish should be terminated 21 days after the onset of spawning in the controls.

32. The second generation (F2) embryos are incubated as described for the F1 embryos and their viability and hatchability assessed.

33. After hatching of the second generation is 90% complete, the number in each incubation chamber is reduced to between 25 and 50 and the larvae released to the growth chambers and treated as the F1 generation until maturity. Larvae are maintained for 28 days post-hatch at which time wet weight and total length is determined.

Observations

Survival

34. Daily assessment of survival is necessary to provide a basis for expression and interpretation of 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 (Ankley et al, 2001; Makynen et al, 2000). In animals 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.

Behavior of adults

35. Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. From the standpoint of existing knowledge of EDCs, alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, may be affected by chemicals that interact with estrogen and/or androgen pathways (Kime et al, 1999; Bayley et al, 1999). Because of the relative subjectivity of this endpoint, it may be necessary to document behavioral alterations via photographs or videotape.

Fecundity

36. Egg production should be determined daily during the spawning phases. In general, the fish usually can be expected to spawn in the early morning (ca. 3 to 4-hours after dawn) so, except for feeding, they should not be disturbed until late morning. This allows time for spawning and fertilization to be completed, and for eggs to water-harden. The spawning substrates or trays can be removed from the tanks to enumerate any eggs that are present. One spawn typically will be comprised of 50 to 150 eggs, however, smaller clutches are not

uncommon depending on the quality of the feeding regime. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity should be expressed on the basis of surviving females per reproductive (test) day per replicate. For example, if all four females survived the treatment in a given replicate for the exposure duration of 21-d, there would be 84 female reproductive days.

Fertilization success

37. After the spawning substrate, tray or eggs have been removed from the tank, the embryos should be separated 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. An alternative to the microscopic approach to determining fertilization success is to enumerate eyed embryos at this time. If the latter approach is used for fertility determination, the embryos should be placed in "incubation" chambers (US EPA, 1982), and held in a system apart from the adults to avoid possible predation. The fertility rate in control animals generally will exceed 90% (Ankley et al, 2001; US EPA, 1987).

Hatchability, larvae appearance, and survival

38. At 25^{o}C, untreated animals will hatch in 3 to 6-d. 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 is typically >95%.

39. The appearance and behavior of hatched larvae can be evaluated, and 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. Survival of the larvae may be assessed through yolk sac absorption (ca., 96 h at 25^{o}C); if estimates of survival are required after this, the animals must be fed live food (generally live *Artemia* nauplii or rotifers) (US EPA, 1982; Hutchinson and Williams, 1994).

Appearance of adults

40. Observations of physical appearance of the adults should be made over the course of the test, and at conclusion of the study. Experience to date with fathead minnows, for example, suggests some EDCs may initially induce changes in the following external characteristics: 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). Notably, chemicals with certain MOA cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone, can cause female fathead minnows to develop pronounced nuptial tubercules (Ankley et al, 2001; Smith, 1974). It also has been reported that estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (Miles-Richardson et al, 1999; Harries et al, 2000). Some morphological variations, in particular coloration, can be equivocal with respect to association with chemical treatment. Specifically, males tend to be dark with vertical banding, but a small number of control females also will exhibit this pattern. Interestingly, in these situations, a hierarchical behavior seems to have developed among females in the test tank, in which a dominant female exhibits male coloration patterns.

41. Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. This type of qualitative assessment might be enhanced through the use of photographs or videotape. Other endpoints, such as the number and size of nuptial tubercles, can be quantified directly or in preserved specimens (Ankley *et al.*, 2001).

Gonad size and morphology, and biochemical endpoints (VTG, steroids)

42. At conclusion of the exposure of the P, F1 and F2 generation, respectively, the fish should be anaesthetized with MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L), wet weighed, and blood collected from the caudal artery/vein with a heparinised microhematocrit capillary tubule. Depending upon size of the fathead minnow (which usually is sex-dependent), collectable blood volumes from adults generally range from 30 to 80 µl (Jensen *et al.*, 2001). Plasma is separated from the blood via centrifugation (3 min; 15,000 g; room temperature), and stored with protease inhibitors at -80^{o}C, until analyzed for vitellogenin and steroids (Korte *et al.*, 2000; Jensen *et al.*, 2001). If whole-body homogenates are used for measurement of vitellogenin (Holbech *et al*., 2001), fish are randomly sampled and frozen in liquid nitrogen. After weighing and homogenization, vitellogenin is analyzed.

Gonad size and morphology

43. After sampling the blood, adult fish should be weighed, and then placed in an appropriate fixative (e.g., 10% buffered formalin) for histological examination. Once fixed, the gonads are removed and wet weighed (to the nearest 0.1 mg) for determination of the gonado-somatic index (GSI=100 × gonad wt/body wt). For example, typical GSI values for reproductively active fathead minnows range from 8 to 13% for females, and 1 to 2% for males (Jensen *et al*., 2001). Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes (Ankley *et al.*, 2001; Makynen *et al.*, 2000).

44. Routine histological procedures can be used to assess condition of testes and ovaries from the fish (Jensen et al, 2001). The fixed gonads should be embedded in paraffin or plastic. Sections should be taken along the long axis of the gonad at 4 to 5 µm intervals, in a serial-step fashion. At a minimum, two serial sections should 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 can be stained with hematoxylin and eosin, and should be evaluated by an experienced pathologist without prior knowledge of the treatment regime associated with specific samples. In general, 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. Classification schemes potentially useful for assessing fathead minnow gonads are described elsewhere (Henderson, 1962; Wallace and Sellman, 1981; Morte and Lacanilao, 198??; Goodbred *et al.*, 1997). Recent studies have documented a variety of alterations in fathead minnow gonadal histology associated with exposure to EDCs with estrogenic or androgenic properties (Ankley *et al.*, 2001; Makynen *et al.*, 2000; Miles-Richardson *et al.*, 1999a, 1999b; Harries *et al.*, 2000).

Vitellogenin

45. Vitellogenin is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of vitellogenin is controlled by interaction of

estrogens, predominantly β-oestradiol, with the estrogen receptor (Kime, 1999). Significantly, males maintain the capacity to produce vitellogenin in response to stimulation with estrogen receptor agonists; as such, induction of vitellogenin in males has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of fish species (Ankley *et al.*, 2001; Harries *et al.*, 2000; Panter *et al.*, 1998, 2002; Kramer *et al.*, 1998; Parks *et al.*, 1999; Tyler *et al.*, 1999, Holbech *et al*., 2001). Different methods are available to assess vitellogenin production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma via enzyme-linked immunoabsorbant assay (ELISA) or radioimmunoassay (RIA).

Sex steroids and thyroid hormone

46. Optionally, plasma concentrations of β-oestradiol, testosterone, 11-ketotestosterone, and/or T3/T4 may be determined using radioimmunoassay (RIA) or other validated techniques optimized for the relatively small sample volumes obtained from all four proposed test species. Reagents necessary for measuring β-oestradiol and testosterone via RIA are available from a variety of commercial and non-commercial sources; however, an antibody for 11-ketotestosterone currently is not commercially available, and radiolabeled 11-ketotestosterone must be prepared via custom synthesis. As such, routine measurement of 11-ketotestosterone as part of this assay would be enhanced by development of readily-accessible (preferably commercial) sources of certain reagents for the RIA.

PERFORMANCE CRITERIA

47. The following criteria for judging data (test) acceptability:

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2;
- There should be documentation (via appropriate analytical chemistry) of purity of the test material, as well as delivery of chemical to the fish (e.g., concentrations of the chemical in test water);
- There should be more than 90% survival of control animals in all test phases over the duration of the chemical exposure, and the control fish in each replicate in the two spawning phases should spawn regularly.
- There should be greater than 80% fertility and hatchability of eggs and embryos, respectively, from the control animals.

DATA REPORTING

Treatment and interpretation of results

48. Different options are available for data analysis; these are comprised primarily of routine hypothesis testing procedures, based on ANOVA, and are described in detail elsewhere. One relatively unique aspect of the experimental design described above concerns collection of spawning data for each test replicate before initiation of chemical exposure. This can be important if there is significant among-tank variation in reproductive output within a treatment that occurs prior to and during the exposure. Analysis of covariance can be used to consider both the pre- and post-exposure data, thereby (potentially) increasing statistical power

of the test to detect biological effects due to the toxicant, versus those associated with the innate characteristics of the cohort of animals in a given experimental unit.

49. It is strongly recommended that a statistician be involved in both the design and analysis of the test results since the test guideline allows for some variation in experimental design.

50. Any endpoints that are significantly affected by the test chemical should be reported as such.

Test report

51. The test report must include the following:

Test substance:

- The report must 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, toxicity to fathead minnow (or other fish species), etc.

Test species:

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

Test conditions:

- The report must specify the conditions under which the test was performed, this include:
  - 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, and also analyses for relevant contaminants (eg PCBs, PAHs and organochlorine pesticides);
  - 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. If the exposure route was by injection, the carrier and injection volume must be stated. If the exposure route was dietary, the food items used to introduce the chemical must be stated.

Results:

- The results must include data for the control (plus solvent control or ip injected control when used) and the treatment fish.
- The table of results must include the mean, standard deviation and range for each test endpoint from the replicates employed in the test. Statistical significance of means should be indicated.

LITERATURE

Andersen, L., Bengtson, B.E., Björk, M., Gessbo, Å., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K.L., Lundgren, A., Petersen, G.I., Steinholz, Å. and Örn, S. (2000) Zebrafish for testing endocrine disrupting chemicals. TemaNord 2000:555. ISBN:92-893-0480-4.

Andersen, L., Gessbo, Å., Holbech, H., Norrgren, L., and Petersen, G. (2002) Effects of exposure during early development to 17 α-ethinylestradiol on the sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology Part C. Accepted.

Ankley GT, Jensen KJ, Kahl MD, Korte JJ, Makynen EA. (2001) Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20: 1276-1290.

American Society for Testing and Materials. (1993) Standard guide for conducting early life-stage toxicity tests with fishes. Method E 1241-92. In *Annual Book of ASTM Standards*. Volume 11.04. Philadelphia, PA, USA, pp 941-967.

Bayley M, Nielsen JR, Baatrup E. (1999) Guppy sexual behavior as an effect biomarker of estrogen mimics. *Ecotoxicol Environ Saf* 43:68-73.

Benoit, DA (1982) User's guide for conducting life-cycle chronic toxicity tests with fathead minnows (*Pimephales promelas*). Environ. Res. Lab. – Duluth, Duluth, MN. EPA 600/8-81-011

Bernet D, Schidmt H, Meier W, Burkhardt-Holm P and Wahli T (1999) Histopathology in fish: proposal for a protocol to assess aquatic pollution. *J Fish Dis* 22: 25-34

Bresch H, Beck H, Ehlermann D, Schlaszus H, Urbanek M (1990) A long-term test comprising reproduction and growth of zebrafish (Danio rerio) with 4-chloroaniline. *Arch Environ Contam Toxicol* 19: 419-427

Environment Canada. (1992). Biological test method: toxicity tests using early life stages of salmonid fish (rainbow trout, coho salmon, or Atlantic salmon). Conservation and Protection, Ontario, Report EPS 1/RM/28, 81 p.

Gast MH, Brungs WA. (1973) A procedure for separating eggs of the fathead minnow. *Prog Fish-Cult* 35:54.

Goodbred SL, Gilliom RJ, Gross TS, Denslow NP, Bryant WL, Schoeb TR. (1997) Reconnaissance of the 17β-estradiol, 11-ketotestosterone, vitellogenin, and gonad histopathology in common carp of United States streams: Potential for contaminant-induced endocrine disruption. Report 96-627. U.S. Geological Survey.

Gray MA and Metcalfe CD (1999). Toxicity of 4-*tert*-octylphenol to early life stages of Japanese medaka (*Oryzias latipes*) *Aquat Toxicol* 46(2):149-154

Groonen S, Denslow N, Manning S, Barnes S, Barnes D and Brouwer M (1999) Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. *Environ Hlth Perspect* 10: 385-390

Hansen DJ and Parrish PR (1977) Suitability of sheepshead minnows (*Cyprinodon variegatus*) for life-cycle toxicity tests. In Aquatic Toxicology and Hazard Evaluation (edited by FL Mayer and JL Hamelink), pp. 117-126, ASTM STP 634

Hansen DJ, Parrish PR, Schimmel SC, and Goodman LR (1978) Life-cycle toxicity test using sheepshead minnows (*Cyprinodon variegatus*). Pp. 1- 116 in Bioassay Procedures for the Ocean Disposal Permit Program. U. S. Environmental Protection Agency, Office of Research and Development. EPA-600/0-78-010

Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, Tyler CR. (2000). Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ Sci Technol* 34: 3003-3011

Henderson NE. (1962) The annual cycle in the testis of the eastern brook trout, *Salvelinus fontinalis* (Mitchill). *Can J Zool* 40:631-645.

Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, and Bjerregaard P. (2001) Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (Danio rerio). Comparative Biochemistry and Physiology Part C 130:119-131.

Hutchinson T H and Williams T D (1994) Culturing of fathead minnow larvae for aquatic toxicity testing: an observational note. *Environ Toxicol Chem,* 13: 665-669.

Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. (2001) Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp Biochem Physiol* 28C(1): 127-141

Kime DE. (1999) *Endocrine Disruption in Fish*. Kluwer Academic Publishers, Norwell, MA, USA.

Korte JJ, Kahl MD, Jensen KM, Pasha MS, Parks LG, LeBlanc GA, Ankley GT. (2000) Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment. *Environ Toxicol Chem* 19:972-981.

Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. (1998) Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. *Aquat Toxicol* 40:335-360.

Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van Der Kraak G, Ankley GT. (2000) Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 48:461-475.

Marte CL, Lacanilao F. (1986) Spontaneous maturation and spawning of milkfish in floating net cages. *Aquaculture* 53:115-132.

Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. (1999) Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 47:129-145.

Miles-Richardson SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP. (1999) Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Environ Res* 80:122-137.

OECD. (2000). Guidance document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23.

Panter GH, Thompson RS, Sumpter JP. (1998) Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat Toxicol* 42:243-253.

Panter GH, Campbell PM, Hutchinson TH, Kime DE, Laenge A, Lye C, McCahon P, Sumpter JP, Tattersfield LJ, Tyler CR. (2002). Effects of endocrine disruptors in the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem*, in press

Panter GH, Tyler CR, Maddix S, Campbell PM, Hutchinson TH, Laenge R, Lye C, Sumpter JP, (1999) Application of an ELISA to quantify vitellogenin concentrations in fathead minnows (*Pimephales promelas*) exposed to endocrine disrupting chemicals. CEFIC-EMSG research report reference AQ001. CEFIC, Brussels, Belgium.

Panter GH, Hutchinson TH, Laenge R, Whale G, Sumpter JP, Tyler CR. (2001) Quantifying gonadal development in sexually maturing fathead minnows (*Pimephales promelas*). CEFIC-EMSG research report reference AQ004. CEFIC, Brussels, Belgium.

Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlanc GA, Sullivan CV. (1999) Fathead minnow (*Pimephales promelas*) vitellogenin: Purification, characterization, and quantitative immunoassay for the detection of estrogenic compounds. *Comp Biochem Physiol* 132C:113-125.

Shioda T and Wakabayashi M (2000) Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*) *Chemosphere* 40(3): 239-243

Smith, RJF. (1974) Effects of 17α-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). *Can J Zool* 52:1031-1038.

Tyler CR, Aerle RV, Hutchinson TH, Maddix S, Trip H. (1999) An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ Toxicol Chem* 18:337-347.

U.S. Environmental Protection Agency. (1982) User's guide for conducting life-cycle chronic toxicity tests with fathead minnows (*Pimephales promelas*). EPA/600/8-81-011. Duluth, MN, USA.

U.S. Environmental Protection Agency. (1987) Guidelines for the culture of fathead minnows *Pimephales promelas* for use in toxicity tests. EPA/600/3-87/001. Environmental Research Laboratory, Duluth, MN, USA.

U.S. Environmental Protection Agency. (1996) Prehatching development of the fathead minnow *Pimephales promelas* Rafinesque. EPA/600/R-96/079. Washington, DC, USA.

Van den Belt K, Verheyen R and Witters H (2001) Reproductiv effects of ethynylestrdadiol and 4t-octylphenol on the Zebrafish (Danio rerio) *Arch Environ Contam Toxicol* 19: 419-427

Wallace RA, Sellman K. (1981) Cellular and dynamic aspects of oocyte growth in teleosts. *Amer Zool* 21:325-343.

West G (1990) Methods of assessing ovarian development in fish: a review. *Aust J Mar Freshwater Res* 41: 199-222

Wester PW and Vos JG (1994) Toxicological pathology in laboratory fish: an evaluation with two species & various environmental contaminants. *Ecotoxicology* 3: 21-44

ANNEX 1

Table 1. Test Conditions For The Fish Reproduction Test Guideline

1. Recommended species	Fathead minnow (*Pimephales promelas*), Medaka (*Oryzias latipes*), Zebrafish (*Danio rerio*), and Sheepshead minnow (*Cyprinodon variegatus*)
2. Test type	Flow-through or semi-static
3Water temperature	25 ± 2°C
4.Illumination quality	Fluorescent bulbs (wide spectrum)
5. Light intensity	10-20 μE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h dark
7. Loading rate	<5 g per L
8. Test chamber size	18L (min): group breeding
9. Test solution volume	10L (min): group breeding
10. Volume exchanges of test solutions	1 - 6 times daily
11. Age of test organisms at initiation	Breeding adults
13. No. of fish per test vessel	Group breeding: 4F + 2M (≥ 6 fish for zebrafish)
14. No. of treatments	≥6 (plus appropriate controls)

ANNEX 1 continued

15. No. vessels per treatment	≥4 (6 preferred)
16. No. of fish per test concentration	Group-breeding: ≥6 (4F + 2 M)
17. Feeding regime	Frozen adult brine shrimp 2x daily
18. Aeration	None unless DO reaches <4.9 mg per L
19. Dilution water	Clean surface, well or reconstituted water; Filtered or reconstituted seawater 25+3ppt salinity for sheepshead minnow
20. Pre- exposure period	≤ 21-days
21. Primary endpoints	Adult survival and behavior, number of spawns, number of eggs/spawn, fertility, secondary sexual characteristics, plasma sex steroids and vitellogenin, gonadosomatic index (GSI) and gonadal histology
22. Optional endpoints	Hatching success, larval development and morphology
23. Test acceptability	Dissolved oxygen ≥60% of saturation; mean temperature of 25 ± 2°C; 90% survival of fish in the controls; successful egg production in controls

ANNEX 2

Table 2. Recommended ranges of water quality characteristics for culturing and testing

Water characteristic	Preferred range	Method of measurement
Temperature (^{o}C)	24.0-26.0 (21.0-23.0 for medaka)	Alcohol or electronic thermometer
Dissolved oxygen (mg/L)	>4.9 mg/L ($\geq$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)	$\leq$5	TOC analyzer
Unionized ammonia (µg/L)	<35	Nesslerization with pH and temperature adjustments