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Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (Fetax)[1]

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1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the developmental toxicity of a test material. The test utilizes embryos of the South African clawed frog, *Xenopus laevis* and is called FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) (**1**).[2] Some of these procedures will be useful for conducting developmental toxicity tests with other species of frogs although numerous modifications might be necessary. A list of alternative anurans is presented in Appendix X1.

1.2 A renewal exposure regimen and the collection of the required mortality, malformation, and growth-inhibition data are described. Special needs or circumstances might require different types of exposure and data concerning other effects. Some of these modifications are listed in Appendix X2 although other modifications might also be necessary. Whenever these procedures are altered or other species used, the results of tests might not be comparable between modified and unmodified procedures. Any test that is conducted using modified procedures should be reported as having deviated from the guide.

1.3 These procedures are applicable to all chemicals either individually or in formulations, commercial products or mixtures that can be measured accurately at the necessary concentrations in water. With appropriate modification these procedures can be used to conduct tests on temperature, dissolved oxygen, pH, physical agents, and on materials such as aqueous effluents (see Guide E 1192), leachates, aqueous extracts of water-insoluble materials, particulate matter, sediment, and surface waters.

1.4 *This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.5 This guide is arranged as follows:

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2. Referenced Documents

2.1 *ASTM Standards:*

D 1193 Specification for Reagent Water[3]

E 380 Practice for Use of the International System of Units (SI)[4]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians[5]

E 943 Terminology Relating to Biological Effects and Environmental Fate[5]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[5]

[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Toxicology.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

[4] *Annual Book of ASTM Standards*, Vol 14.02.

[5] *Annual Book of ASTM Standards*, Vol 11.04.

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates and Amphibians[5]

3. Terminology

3.1 *Descriptions of Terms Specific to This Standard:*

3.1.1 The words "must," "should," "may," "can," and "might," have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.1.2 A developmental toxicant is a test material that affects any developmental process. Therefore, a developmental toxicant affects embryo mortality and malformation, and causes growth inhibition. A teratogen is a test material that causes abnormal morphogenesis (malformation). The Teratogenic Index or TI is a measure of developmental hazard (**1**). TI values higher than 1.5 signifies larger separation of the mortality and malformation concentration ranges and, therefore, a greater potential for all embryos to be malformed in the absence of significant embryo mortality. The TI is defined as the 96-h LC50 divided by the 96-h EC50 (malformation).

3.1.3 For definitions of other terms used in this guide, refer to Guides E 729 and E 1023, also Terminology E 943. For an explanation of units and symbols, refer to Practice E 380.

4. Summary of Guide

4.1 In FETAX, a range-finding and three replicate tests are performed on each test material. A control in which no test material has been added is used to provide 1) a measure of the acceptability of the test by indicating the quality of embryos and the suitability of the FETAX solution, test conditions and handling procedures, and 2) a basis for interpreting data from other treatments. Each test consists of several different concentrations of test material with two replicate dishes of each concentration. Each of the three tests is conducted using embryos from a different male/female pair of *Xenopus laevis*. A reference toxicant (6-aminonicotinamide) should be used as a quality control measure. The 96-h LC50 and 96-h EC50 (malformation) are determined by probit analysis and the TI (Teratogenic Index) is calculated by dividing the LC50 by the EC50. Growth inhibition is determined by measuring the head-tail length of each embryo and determining whether growth at a particular concentration is significantly different from that of the control. Other useful data can be collected (for example, pigmentation, locomotion, and hatchability) to expand the utility of the test.

5. Significance and Use

5.1 FETAX is a rapid test for identifying developmental toxicants. Data may be extrapolated to other species including mammals. FETAX might be used to prioritize samples for further tests which use mammals. Validation studies using compounds with known mammalian or human developmental toxicity, or both, suggest that the predictive accuracy will approach or exceed 85 % (**2**). The accuracy rate compares favorably with other currently available "*in vitro* teratogenesis screening assays" (**3**). Any assay employing cells, parts of embryos, or whole embryos other than *in vivo* mammalian embryos is considered to be an *in vitro* assay.

5.2 It is important to measure developmental toxicity because embryo mortality, malformation, and growth inhibition can often occur at concentrations far less than those required to affect adult organisms.

5.3 Because of the sensitivity of embryonic and early life stages, FETAX provides information that might be useful in estimating the chronic toxicity of a test material to aquatic organisms.

5.4 Results from FETAX might be useful when deriving water quality criteria for aquatic organisms (**4**).

5.5 FETAX results might be useful for studying structure-activity relationships between test materials and for studying bioavailability.

6. Safety Precautions

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and safety glasses, and using pipets to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers and the use of fume hoods, should be taken when conducting tests on volatile materials. Information provided in Material Safety Data Sheets on toxicity to humans (**5**), recommended handling procedures (**6**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (**7**) and with test materials that are, or are suspected of being, carcinogenic (**8**).

6.2 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

6.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a fume hood.

6.4 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

6.5 Because FETAX solution and test solutions are usually good conductors of electricity, use of ground fault

systems and leak detectors should be considered to help avoid electrical shocks.

7. Apparatus

7.1 *Facilities for Maintaining and Breeding Xenopus*—Adults should be kept in an animal room that is isolated from extraneous light which might interfere with a consistent photoperiod of 12-h day/12-h night. The role that circadian rhythm plays in *Xenopus* reproduction has not been investigated. A consistent photoperiod is therefore recommended so that *Xenopus* can be bred year-round. Adults can be kept in large aquaria or in fiberglass or stainless steel raceways at densities of 4 to 6 per 1800 cm^2 of water surface area. The sides of tanks should be opaque and at least 30 cm high. The water depth should be between 7 and 14 cm. Water temperature for adults should be 23 ± 3°C.

7.1.1 Two types of breeding aquaria have been used successfully. A 5 or 10-gal glass aquarium may be used if fitted with a 1-cm mesh suspended about 3-cm from the bottom of the aquarium so that deposited eggs will lie undisturbed on the bottom of the aquarium. Hardware cloth or other metal mesh must not be used. Nylon or plastic mesh is recommended. The sides of the breeding aquarium should be opaque and a bubbler fitted to oxygenate the water. The top of the aquarium should be covered with an opaque porous material such as a fiberglass furnace filter. Alternatively, an adequate breeding tank can be constructed from two plastic dish pans (at least 38 by 38 cm) stacked one in the other. The floor of the topmost pan is perforated. A corkborer can be used to create 1.5-cm holes for the eggs to fall through.

7.2 *Facilities for Conducting FETAX*—A constant temperature room or a suitable incubator is required although a photoperiod is unnecessary. The incubator must be capable of holding 24 ± 2°C. Abnormal development will occur at temperatures greater than 26°C. Covered 60-mm glass Petri dishes should be used as test chambers except that disposable 55-mm polystyrene Petri dishes should be used if a substantial amount of the test material binds to glass but not to polystyrene. A binocular dissection microscope capable of magnifications up to 30× is required to count and evaluate abnormal embryos. A simple darkroom enlarger is used to enlarge embryo images 2 to 3 times for head-tail length measurements. It is also possible to measure embryo length through the use of a map measurer or an ocular micrometer. However, the process is greatly facilitated by using a digitizer interfaced to a microcomputer. The microcomputer is also used in data analysis.

7.3 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or water in which embryos will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that would adversely affect embryo growth or development. Additionally, items that contact stock solutions or test solutions should be chosen to minimize sorption of most test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastic should be used whenever possible to minimize dissolution, leaching, and sorption. Rigid plastics may be used for holding, acclimation, and in the water supply system, but they should be soaked for a week before use in water used for adult maintenance.

7.3.1 FETAX solution, stock solutions, or test solutions should not contact brass, copper, lead, galvanized metal, or natural rubber before or during the test. Items made of neoprene rubber or other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect either survival or growth of the embryos and larvae of the test species.

7.4 *Cleaning*—At the end of each test, all glass dishes and other glassware that are to be used again should be immediately emptied, rinsed with water, and cleaned by the following procedure.

7.4.1 *Glassware Washing Procedure:*

7.4.1.1 Soak 15 min, and scrub with tissue culture compatible detergent in tap water.

7.4.1.2 Rinse twice with tap water.

7.4.1.3 Rinse once with fresh, dilute (10 %, v/v) hydrochloric acid to remove scale, metals, and bases.

7.4.1.4 Rinse twice with tap water.

7.4.1.5 Rinse once with full strength reagent-grade[6] acetone to remove organic compounds.

7.4.1.6 Rinse well with hot tap water.

7.4.1.7 Rinse well with distilled water or FETAX solution.

7.4.1.8 Heat the glassware in an oven at 350°C for 3 h to drive off any residual acetone. Toxicity problems have occurred in past experiments when this glassware washing procedure was omitted.

7.5 *Acceptability*—Before FETAX is conducted in new test facilities it is desirable to conduct a "non-toxicant" test, in which all test chambers contain FETAX solution with no added test material. The embryos should grow, develop, and survive in numbers consistent with an acceptable test (see 14.1). The magnitude of the chamber-to-chamber variation should be evaluated.

8. Water for Culturing *Xenopus* Adults

8.1 *Requirements*—Besides being available in adequate supply, the water should (a) allow satisfactory survival and reproduction of the adults, (b) be of uniform quality, and (c) not necessarily affect results of the test.

8.2 *Source:*

8.2.1 Natural water is preferred for adult culture. It should be obtained from an uncontaminated source that provides uniform quality. The quality of water from a well or spring is usually more uniform than that of a surface water. If a surface water is used as a source of fresh water, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. Water temperature should be adjusted to 23 ± 3°C before being used to culture adults.

8.2.2 Dechlorinated water can be used to culture adults as long as residual chlorine and its oxidants are reduced to levels that do not affect survival and reproduction. Dechlorinated water should only be used as a last resort because dechlorination is often incomplete. Sodium bisulfite

[6] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Reagent Chemicals and Standards," by Joseph Rosin, D. Van Nostrand Co., Inc., New York, NY, and the "United States Pharmacopeia."

is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines (9). Fluorides can be removed by passage over activated alumina columns (10). In addition to residual chlorine, chloramines, and fluoride, municipal drinking water often contains unacceptably high concentrations of copper, lead, and zinc, and quality is often rather variable. Excessive concentrations of most metals can usually be removed with a chelating resin (11).

8.3 *Treatment:*

8.3.1 Water for culturing adults should be aerated by the use of air stones or surface aerators. Air used for aeration should be free of fumes, oil, and water. Compressed air supplies might be contaminated with oil or water containing rust or sludge. Some compressed air supplies might also have a high level of carbon monoxide. A low-pressure blower will provide high-quality air without the problems associated with a high-pressure air supply as long as its air supply is uncontaminated. Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. However, it is not absolutely necessary to aerate the water for *Xenopus* adults.

8.3.2 Filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low and as a pretreatment before filtration through a finer filter. Organics may be removed by filtration through activated charcoal filtration. Charcoal filters should be changed on a monthly basis.

8.3.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (12) equipped with an intensity meter and flow controls, or passed through a filter with a pore size of 0.45 μm or less.

8.4 *Characterization:*

8.4.1 The following items should be measured at least quarterly: pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, ammonia, bromide, beryllium, cadmium, chromium, copper, iron, lead, manganese, mercury, nickel, selenium, silver, and zinc. For each method used the detection limit should be below (a) the concentration in the water or (b) the lowest concentration that has been shown to adversely affect the test species.

8.4.2 Physical and chemical limits on water: pH should be between 6.5 and 9 (13). The TOC should be less than 10 mg/L, while alkalinity and hardness both should be between 16 and 400 mg/L as $CaCO_3$ (14). Table 1 shows the recommended maximum concentrations for some contaminants that have often been found to be in excess concentration in laboratory water supplies. The values reported are one tenth of the minimum concentration that inhibits growth. While these data are not indicative of the effect of long-term exposure of adults on reproductive success, they, nonetheless, serve as a guide for limiting adult exposure to these metals. The maximum quantity of the other contaminants listed in 8.4.1 should meet EPA freshwater chronic water quality criteria (13).

TABLE 1 Recommended Maximum Concentrations of Some Metals

Metal[A]	Recommended Maximum Concentration (μg/L)
Cadmium (2)	10.0
Lead (2)	5.0
Mercury (2)	0.144
Nickel (2)	25.0
Selenium (unpublished)	140.0
Zinc (2)	70.0

[A] Tested in FETAX at 100 mg/L hardness as $CaCO_3$. Values reported are one tenth of the minimum concentration to inhibit growth.

9. FETAX Solution Water

9.1 *Requirements*—FETAX solution should be used for breeding and static or renewal assays. FETAX solution should also be used for flow-through experiments whenever possible. However, should the need for a large volume preclude the use of FETAX solution, then water conforming to the specifications listed in Section 8 may be used. The water must allow embryonic growth at the same rate as FETAX solution and there should be no differences between control mortality and malformation rates.

9.2 *Formulation*—FETAX solution is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per litre of deionized or distilled water. The pH of the final solution should be 7.6 to 7.9. All chemicals should be reagent-grade[6] or better. Deionized or distilled water must conform to Type I ASTM water (Specification D 1193).

10. Test Material

10.1 *General*—The test material should be reagent-grade[6] or better unless a specific test involves an unknown complex mixture, formulation, commercial product, or technical-grade or use-grade material. Before a test is begun, the following should be known about the test material:

10.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material.

10.1.2 Solubility and stability in water.

10.1.3 Estimate of toxicity to humans.

10.1.4 Recommended handling procedures (see Section 6).

10.1.5 For unknown samples much of the information specified in 10.1.1 through 10.1.4 will be lacking, but the pH, hardness, alkalinity, and conductivity of the sample should be measured.

10.2 *Stock Solution:*

10.2.1 In most cases the test material can be added directly to the FETAX solution in the Petri dishes but usually it is dissolved in a solvent to form a stock solution. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. Stock solutions should be prepared daily unless analytical data indicate the solution is stable with time. If the test material is subject to photolysis, the stock solution should be shielded from light.

10.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is FETAX solution. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such acid or base might affect the

pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols or organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of minimum necessary amount of a strong acid or base. Any adjustments of pH can send the test material through a transition to affect changes in such properties as solubility or degree and type of dissociation, or both. Prior to testing, as much chemical and physical data as are available on the test material should be obtained and considered prior to making decisions on pH adjustments.

10.2.2.1 If a solvent other than FETAX solution is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect *Xenopus* embryo growth and survival. Because of its low toxicity, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as dimethyl sulfoxide and acetone also may be used as solvents. Concentrations of triethylene glycol, dimethyl sulfoxide, and acetone in test solutions should be <1.6 %, <1.1 %, <1.1 % v/v respectively. These concentrations have been found not to cause any adverse effects in FETAX (**15**). At times, concentrations approaching 1 % solvent are necessary to keep test materials in solution for FETAX. This is often the case when the assay is used in testing pure compounds for, the purpose of comparing test results with mammalian data. If possible, it is desirable to perform the test using two different solvents and compare the results. This will help in identifying possible interactions between a solvent and test material.

10.2.2.2 Ethanol is not recommended because its teratogenic index (TI) in FETAX is approximately 1.4. Methanol has high toxicity in FETAX. Acetone might stimulate the growth of microorganisms and is quite volatile. If an organic solvent is used it should be reagent-grade[6] or better. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions.

10.2.3 If a solvent other than dilution-water or FETAX solution is used, (a) at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test and (b) a dilution-water or FETAX solution control should be included in the test. If no solvent other than dilution-water or FETAX solution is used, then a dilution-water or FETAX solution control must be included in the test.

10.2.3.1 The concentration of solvent must be the same in all test solutions that contain test material and the solvent control must contain the same concentration of solvent.

10.2.3.2 If the test contains both a dilution-water or a FETAX-solution control and a solvent control, the mortality, malformation, and growth inhibition should be compared using a two-tailed t-test. If a statistically significant difference in either mortality, malformation, or growth inhibition is detected between the two controls, only the solvent control may be used as the basis for comparison in the calculation of results.

10.2.3.3 If a solvent other than dilution-water or FETAX solution is used to prepare a stock solution, it might be desirable to conduct simultaneous tests using chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvents on results of the test.

11. Test Organisms

11.1 *Species*—FETAX is designed to use embryos of the South African clawed frog *Xenopus laevis* (Daudin). Information regarding the basic biology and development of this species has been reported by Deuchar (**16, 17**). Appendix X1 lists other North American species that can be used in situations where *Xenopus* cannot, although there will be differences in the rate of development and the method of inducing breeding. Many anurans only breed in a specific season during the year. The length of exposure might have to be altered to allow proper organogenesis.

11.2 *Source*—For breeding, adult frogs may be obtained from various supply houses or independent suppliers. Proven breeders should be requested from the supplier. Each animal should be thoroughly examined upon arrival for skin lesions or red patches on the ventral surfaces. Skin lesions are indicative of nematode infection while the red patches indicate *Aeromonas* infection. Care should be taken to ensure that only healthy, sexually mature frogs are placed in the colony.

11.3 *Adults:*

11.3.1 *Selection—Xenopus* males should be 7.5 to 10 cm in crown-rump length and at least 2 years of age. Males have dark arm pads on the underside of each forearm and lack cloacal lips. Females should be 10 to 12.5 cm in length and at least 3 years old. Females are always larger than males and easily identified by the presence of fleshy cloacal lips.

11.3.2 *Diet*—The minimum recommended diet for adults should be three feedings per week of ground adult beef liver. Liquid multiple vitamins should be added to the ground beef liver. The concentration of vitamins is shown in Table 2. Concentrations of vitamins from 0.05 to 0.075 cm^3/5 g liver are appropriate. All food should be screened for the test material if the test material is present in the environment. All liver beef must meet USDA standards for human consumption.

11.3.3 *Temperature*—Adults should be kept at 23 ± 3°C.

11.3.4 *Circadian Rhythm*—Adults should be kept on a 12-h day/12-h night cycle. The role that circadian rhythm plays in *Xenopus* reproduction has not been investigated. Thus, a consistent photoperiod should be maintained.

11.4 *Breeding*—Males and females are bred as a single pair. The frogs should be bred in the same water in which the test is to be conducted. To induce breeding, the male and the female should receive 250 to 500 and 500 to 1000 IU, respectively, of human chorionic gonadotropin by way of

TABLE 2 Recommended Concentration of Vitamins[A]

Vitamin A, IU	1500
Vitamin D, IU	400
Vitamin E, IU	5
Vitamin C, mg	35
Thiamine, mg	0.5
Riboflavin, mg	0.6
Niacin, mg	8
Vitamin B_6, mg	0.4
Vitamin B_{12}, mcg	2

[A] Per millilitre of solution.

injection into the dorsal lymph sac. The hormone concentration should be 1000 IU/mL in sterile 0.9 % NaCl. A 1-mL tuberculin syringe fitted with a ½-in. long, 26-gage needle should be used to make the injection. Larger bore needles might allow leakage of hormone from the injection site. The amount of human chorionic gonadotropin injected depends on the time of year and condition of the adults. Lower doses are usually used in spring and higher doses in fall. Amplexus normally ensues within 2 to 6 h and egg deposition about 9 to 12 h after injection. The eggs should be immediately inspected for fertility and quality. The fertility rate should be >75 %. Eggs laid in "strings" or not perfectly round should not be used because they develop abnormally.

11.5 *Embryos:*

11.5.1 *Removal of Jelly Coat*—Dejellying of embryos should begin immediately following the end of egg laying. Dejellying of embryos should be carried out by gentle swirling for 1 to 3 min in a 2 % w/v L-cysteine (CAS #52-90-4) solution prepared in FETAX solution. The cysteine solution should be adjusted to pH 8.1 with 1 N NaOH. The solution should be made up immediately prior to use. Dejellying should be monitored continuously and the process stopped just after all jelly is removed. Care should be taken not to treat the embryos too long because survival will be reduced.

11.5.2 *Staging of Embryos*—Nieuwkoop and Faber must be used in all staging of embryos **(18)**.

11.5.3 *Embryo Selection*—Normally cleaving embryos must be selected for use in testing. The "Atlas of Abnormalities" [7] should be consulted in order to determine which embryos are normal. It is best to use two levels of selection. In double selection, normally cleaving embryos are first sorted into dishes containing fresh FETAX solution. After a short period during which cleavage continues, embryos are again sorted ensuring that only normal embryos are selected. Abnormal pigmentation should be viewed as an indicator of bad embryos. Either Nieuwkoop and Faber **(18)** or the "Atlas of Abnormalities" can be used as a reference to determine whether the cleavage pattern is normal. Midblastula (stage 8) to early gastrula (stage 11) must be used to start the test. Embryos chosen prior to stage 8 might develop abnormal cleavage patterns later whereas embryos selected after stage 11 have commenced organogenesis. A large bore blood bank Pasteur pipet can be used to transfer embryos at this stage without harm. The sorting should be done in 100-mm Petri dishes.

12. Procedure

12.1 *Experimental Design*—FETAX is a 96-h renewal whole embryo assay that can be used to evaluate the developmental toxicity of a test material. Exposure is continuous throughout the test. For each concentration two dishes each containing 25 embryos and 10 mL of test solution are used. For each control, four dishes of 25 embryos each are used. Embryos must be randomly assigned to test dishes. Dishes must be randomly assigned to their positions in the incubator. In order to properly evaluate developmental toxicity, mortality, malformation, and growth-inhibition, data must be collected. In most tests it will be possible to generate concentration-response curves for mortality, malformation, and growth inhibition. The mortality and malformation concentration-response curves should then be used to estimate the concentration that would affect 50 % of the exposed embryos. At least 90 % of the FETAX-solution controls must have attained stage 46 at 96 h **(18)**.

12.2 *Temperature and pH Requirements:*

12.2.1 *Temperature*—24 ± 2°C must be maintained throughout the 96-h test. Temperatures higher than 26°C cause malformation whereas low temperatures prevent the controls from reaching stage 46 in 96 h.

12.2.2 *pH*—The pH of the stock and test solutions should be 7.7 and must be between 6.5 and 9.0 **(13)**. The pH of a control dish and the pH of the highest test concentration should be measured at the beginning of the test and every 24 h thereafter to determine if they have changed.

12.3 *Beginning the Test:*

12.3.1 *Range-Finding*—A range-finding test is recommended. The range-finding test should consist of a series of at least seven concentrations that differ by a factor of ten. This should be adequate to delineate the concentration range needed to establish the 96-h LC50 and EC50 (malformation). The greater the similarity between the range-finding and replicate-definitive test, the more useful the range-finding test will be. Growth inhibition data are not collected from range-finding tests.

12.3.2 *Replicate-Definitive Tests:*

12.3.2.1 *Number of Tests and Data Collection*—Three definitive tests should be conducted on each test material in a random block design. Each test consists of a geometric concentration series (dilution factor = 0.5). Because it is necessary to acquire data on mortality, malformation, and growth inhibition, the concentration series needs to be adjusted to the expected 96-h LC50, 96-EC50 (malformation), and the minimum concentration needed to inhibit growth (MCIG). To ensure an adequate supply of normal embryos for each test, three mating pairs should be induced and clutches harvested separately. Embryos should be sorted to ensure viability prior to testing. Each test uses early embryos derived from a single mating pair; if the controls from a particular mating pair indicate a problem with fertility or viability of early embryos, the test will be unacceptable for that particular clutch. Each individual test will yield data that will be used to generate concentration-response curves for mortality, malformation, and growth inhibition.

12.3.2.2 *Maintenance of Separate Clutches*—It is necessary to keep clutches separate because embryos from a particular mating pair might develop poorly although they initially appear acceptable. This would cause all the embryos to be discarded if embryos are mixed from different mating pairs.

12.3.3 *Experimental Dilutions*—Each test should consist of at least five concentrations for determining concentration-response curves for both mortality and malformation. At least three concentrations should be within 16 to 84 % effect on the mortality and malformation concentration-response curves. Each test should consist of two dishes for each

[7] Available from John A. Bantle, Dept. of Zoology, 430 LSW, Oklahoma State University, Stillwater, OK 74078.

concentration of test material and four dishes for each control.

12.3.3.1 Each test should be performed with embryos derived from a single mating pair regardless of the number of replicate dishes. The selection of experimental design and statistical methods required to evaluate mortality, malformation, and growth-inhibition data, should consider the type of compound or chemical mixture being evaluated and the limitations that sample or time availability might imply as far as appropriate statistical techniques (**19**).

12.3.4 *Reference Toxicant*—For a positive control or reference toxicant, 6-aminonicotinamide presents a mortality and malformation data base convenient for reference purposes. Commercial sources for the 6-aminonicotinamide (CAS #329-89-5; formula weight, 137.14) should specify the physicochemical data and the purity for the compound which ensure its being comparable to that readily available to other laboratories (for example, UV spectroscopic characterization: at A_{257} and pH 1.8, 6-aminonicotinamide has an extinction coefficient of 13.9 mm and an absorbance ratio (A_{257}/A_{302}) of 2.28). The purity should be >99 %. From this published data base for 6-aminonicotinamide, the 96-h LC50 is 2500 mg/L (95 % CI = 2350 to 2650) and the 96-h EC50 for malformation is 5.5 mg/L (95 % CI = 3.9 to 6.9), or a Teratogenic Index of 455 (**20**). For each test, the positive control consists of exposing two dishes of 25 embryos each to 2500 mg 6-aminonicotinamide/L and two dishes of 25 embryos each to 5.5 mg 6-aminonicotinamide/L. The mortality and malformation observed should be between 40 and 60 %. For example, at 2500 mg/L, 20 to 30 of the 50 embryos should have died by 96 h. Only those biological responses related to mortality and malformation are considered in this analysis; growth inhibition is not considered in regard to responses to 6-aminonicotinamide. If results with the reference toxicant are inconsistent or at variance with previous experience, the source of the difference and the influence on test results should be considered.

12.4 *Renewal*—The renewal procedure should be used for the standard FETAX test. The renewal procedure entails fresh replacement of test material every 24 h during the test. Just prior to this change it is advisable to measure the pH of the control and the highest test dishes in order to determine if significant changes occurred. Renewal should be accomplished by removing the test solution with a Pasteur pipet. The orifice of the Pasteur pipet should be enlarged and fire-polished to accommodate embryos without damage in case the embryos are accidentally picked up. This procedure should proceed quickly in order to minimize embryo desiccation. This is the standard procedure for FETAX but two other variants are allowed as described in the Appendix X2. Variations to the renewal procedure must be reported.

12.5 *Duration of the Test*—The standard exposure time for FETAX is 96 h and the attainment of stage 46 in controls. Deviations from this standard exposure time must be reported as deviating from standard FETAX conditions.

12.6 *Biological Data:*

12.6.1 *Mortality*—Dead embryos must be removed at the end of each 24-h period during the 96-h test at the time solutions are changed. If dead embryos are not removed, then microbial growth can occur that might kill live embryos. Death at 24 h (stage 27) is ascertained by the embryo's skin pigmentation, structural integrity, and irritability. At 48 h (stage 35), 72 h (stage 42), and 96 h (stage 46) the lack of heartbeat serves as an unambiguous sign of death. At 96 h of exposure or stage 46 of controls, the number dead is recorded. Dead embryos are removed and the remaining live embryos fixed in 3 % formalin.

12.6.2 *Malformation*—Malformations must be recorded at the end of 96 h. The "Atlas of Abnormalities" should be used in scoring malformations, particularly slight malformations. Embryos exposed to the test material should also be compared to appropriate controls. The number of malformations in each category should be reported in standard format for ease of interlaboratory comparison (Fig. 1).

12.6.3 *Growth Inhibition*—The ability of a material to inhibit embryonic growth is often the most sensitive indicator of developmental toxicity. Head-tail length data (growth) must be collected at the end of each test. If the embryo is curved or kinked, then the measurement should be made as if the embryo were straight. In other words the contour of the embryo should be followed (see 7.2). Measurement should be made after embryos are fixed in 3 % formalin. No significant length reductions due to formalin fixation have been observed. The minimum concentration to inhibit growth (MCIG) is the minimum concentration of test material that significantly inhibits growth as determined by measurement of head-tail length. A significant difference in growth should be determined by the t-Test for grouped observations at the $p = 0.05$ level (**20**).

12.6.4 *Additional Data*—Different types of data have been collected in FETAX and may, at the user's option, be used in addition to the mortality, malformation, and growth inhibition data listed above (see Appendix X2).

13. Analytical Methodology

13.1 The methods used to analyze test solutions might determine the usefulness of the test results if the results are based on measured concentrations. For example, if the analytical method measures any impurities or reaction or degradation products along with the parent test material, results can be calculated only for the whole group of materials, and not for the parent material by itself, unless it is demonstrated that such impurities and products are not present.

13.2 If samples of stock solutions or test solutions cannot be analyzed immediately, they should be handled and stored appropriately (**21**) to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

13.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist, methods should be obtained from other reliable sources (**22**).

14. Acceptability of Test

14.1 A test using embryos from a single mating pair should be considered unacceptable if one or more of the following occurred.

14.1.1 Hardware cloth or metal mesh was used as a support in the breeding aquarium.

FIG. 1 Scoresheet of Malformations at 96 h

Directions: Place a check in each box for each type of malformation. The resultant scoresheet reads like a histogram.

INVESTIGATOR _____ COMPOUND _____

DATE ____/____/____ CONCENTRATION _____

TOTAL SURVIVING _____ TEST # _____

% MALFORMED _____ DISH # _____

Malformations:	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
Severe																									
Stunted																									
Gut																									
Edema (multiple)																									
a. cardiac																									
b. abdominal																									
c. facial																									
d. cephalic																									
e. optic																									
Axial malformations																									
A. tail																									
B. notocord																									
C. fin																									
Face																									
Eye																									
Brain																									
Hemorrhage																									
Cardiac																									
Blisters																									
Other (specify)																									

TOTAL SURVIVING _____ TEST # _____

% MALFORMED _____ DISH # _____

Malformations:	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
Severe																									
Stunted																									
Gut																									
Edema (multiple)																									
a. cardiac																									
b. abdominal																									
c. facial																									
d. cephalic																									
e. optic																									
Axial malformations																									
A. tail																									
B. notocord																									
C. fin																									
Face																									
Eye																									
Brain																									
Hemorrhage																									
Cardiac																									
Blisters																									
Other (specify)																									

14.1.2 In the controls, either the mean survival is <90 % or the mean malformation in embryos is >7 %, or both.

14.1.3 If 90 % of the FETAX-solution-only controls do not reach stage 46 by the end of 96 h. The primary cause of control embryos not reaching stage 46 is low temperature (see 12.2.1).

14.1.4 If dilution water was used in the test, it did not allow embryonic growth at the same rate as FETAX solution.

14.1.5 The deionized or distilled water does not conform to Type I ASTM standard.

14.1.6 A required dilution-water or FETAX solution control or solvent control was not included in the test.

14.1.7 The concentration of solvent was not the same in all treatments, except for a dilution-water or FETAX-solution control.

14.1.8 Staging of embryos was performed using a reference other than Nieuwkoop and Faber **(18)**.

14.1.9 The test either was started with less than stage 8 blastulae or greater than stage 11 gastrulae.

14.1.10 All Petri dishes were not physically identical throughout the test.

14.1.11 Petri dishes were not randomly assigned to their positions in the incubator.

14.1.12 The embryos were not randomly assigned to the Petri dishes.

14.1.13 Required data concerning mortality, malformation, and growth were not collected.

14.1.14 The pH of the test solution was <6.5 or >9.0 in the control or highest test concentration.

14.1.15 Dead embryos were not removed after each 24-h (±2 h) interval.

14.1.16 Consistently deviating from the temperature limits as stated in 12.2.1. A short-term deviation of more than ±2°C might be inconsequential.

14.1.17 If the reference toxicant was used, there were <20 or >30 of 50 embryos killed in the 2500 mg 6-aminonicotinamide/L positive control or <20 or >30 of 50 embryos malformed at 5.5 mg 6-aminonicotinamide/L, or both.

15. Documentation

15.1 The record of the results of an acceptable FETAX should include the following information either directly or by reference to existing publications.

15.1.1 Name of test material, investigator(s) name, location of laboratory, and dates of initiation and termination of test.

15.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used. For some complex environmental mixtures a great deal of this information might be lacking.

15.1.3 If a dilution water other than FETAX solution is used, its chemical characteristics and a description of any pretreatment.

15.1.4 Recent analyses of FETAX solution and adult culture water.

15.1.5 pH measurements of control and of the highest test concentrations at the end of each 24-h time period. Available data on sample hardness, alkalinity, conductivity, total organic carbon (TOC), concentration of dissolved oxygen, and metal content.

15.1.6 The mortality, malformation rates, and the mean embryo length at 96 h in the dilution-water, FETAX solution, or solvent control.

15.1.7 The mortality and malformation results obtained for the 6-aminonicotinamide positive control. If a full concentration-response curve was performed, then the 96-h LC50, the 96-h EC50 (malformation), and their confidence limits should be reported.

15.1.8 The 96-h LC50, the 96-h EC50 (malformation), the TI (96-h LC50/96-h EC50 (malformation)), and the minimum concentration to inhibit growth (MCIG) for each test. The geometric means of these values and their 95 % confidence limits. Concentration-response data for mortality, malformation, and growth inhibition may be provided.

15.1.9 A table for each test that lists the percent mortality, percent malformation, and the head-tail length at each concentration tested.

15.1.10 The names of the statistical tests employed, the alpha-levels of the tests, and some measure of the variability of the hypothesis tested.

15.1.11 The types, frequency, and severity of malformations. The types of malformations and their severity might differ over the different concentrations tested. It might be best to define ranges of concentrations tested and create a summary table that lists the malformations that occurred in each concentration range.

15.1.12 Any deviations from standard FETAX (see Appendixes X1 and X2).

16. Keywords

16.1 amphibia; developmental toxicity; FETAX; screening test; short-term chronic test; teratogenicity; *Xenopus*

APPENDIXES

(Nonmandatory Information)

X1. LIST OF ALTERNATIVE SPECIES

X1.1 *Use of Alternative Species*—Although FETAX was designed expressly for the use of *Xenopus laevis*, it might be necessary to use endemic species when required by regulations or other considerations. Users are cautioned that many endemic species of frogs are threatened by pollution and habitat loss and the user should carefully consider the

environmental consequences of large-scale collection of local anuran species. Deviations from standard procedures must be reported (see Section 15) and it will be difficult to compare data between standard FETAX and data derived using an alternative species.

X1.2 *Recommended Anurans*—Members of the family Ranidae (for example, *Rana pipiens*) and Bufonidae (for example, *Bufo fowleri*) might be best suited for FETAX, because the number of eggs or the seasonal availability, or both, are more limited for other species. Seasonal availability can be extended by 2 to 3 months using human chorionic gonadotropin injection. *Rana catesbiena* and *Bufo americanus* are likely as well suited as *Rana pipiens* and *Bufo fowleri* **(23)**. High egg production, geographical range, short hatching periods, and other factors would indicate that these four species could serve as alternatives. Comparative sensitivities to inorganic mercury have been reported for some of these species **(23)**. These studies have reported a range in sensitivity to inorganic mercury which should be taken into account when comparing data with other amphibian species.

X2. ADDITIONAL DATA AND ALTERNATIVE EXPOSURES

X2.1 *Additional Data*—Other types of data can be collected in FETAX that increases its versatility. The types of data listed below represent some that have been collected in past experiments. In the case of pigmentation and locomotion, scoring is subjective.

X2.1.1 *Pigmentation*—Collecting data on pigmentation might be useful for measuring neural damage because it is thought that the size of the pigment patches is under nervous control. Agents that affect these nerves cause smaller pigment patches and the overall color of the 96-h larvae will pale. Comparison to the standard "Atlas of Abnormalities" and suitable controls must be made in order to determine abnormal pigmentation. Other causes of depigmentation are possible including loss of melanin production. A concentration-response curve can be generated and an EC50 (pigmentation) determined.

X2.1.2 *Locomotion*—Collecting locomotion data is potentially useful in measuring specific neural or muscle damage since larvae with substantial cellular damage swim poorly or erratically. The ability to swim properly should be determined by comparison to appropriate controls. A concentration-response curve can be generated and an EC50 (locomotion) determined.

X2.1.3 *Hatchability*—The embryos hatch from the fertilization membrane between 18 and 30 h. The number failing to hatch at 48 h should be recorded. Delay or failure indicates a slowing of developmental processes. This is analogous to staging the embryos at the end of the 96-h time period except that it is much easier to score hatching. A concentration-response curve can be generated and a EC50 (hatching) determined.

X2.2 *Additional Exposures:*

X2.2.1 *Additional Exposure Length*—In special circumstances, exposure periods exceeding 96 h or pulse exposures, or both, may be performed. Data so collected should be reported as deviating from standard FETAX.

X2.2.2 *Static*—In the static technique, the test material is added at the beginning of the test and not changed. It should be recognized that many test materials will undergo loss in a short period of time. The static technique should only be used for materials that are extremely stable and do not volatilize or sorb to the test dishes. The cost or the size of the sample might also dictate that the static technique be used. This variation in procedure must be reported as deviating from standard FETAX.

X2.2.3 *Flow-Through*—A toxicant-delivery system is used to continuously deliver toxicant and dilution water to the embryos. Small glass containers with bottom screening are used to contain the embryos in a larger diluter apparatus. The flow-through technique is recommended for chemicals that degrade quickly or are volatile or for large volume environmental samples. Every attempt should be made to use FETAX solution as the diluent. This variation in procedure must be reported as deviating from standard FETAX.

REFERENCES

(1) Dumont, J., Schultz, T. W., Buchanan, M., and Kao, G., "Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)—A Short-term Assay Applicable to Complex Environmental Mixtures," In: *Short-term Bioassays in the Analysis of Complex Environmental Mixtures III*, Waters, Sandhu, Lewtas, Claxton, Chernoff and Nesnow, eds., Plenum, New York, NY, 1983, pp. 393–405.

(2) Dawson, D. A., and Bantle, J. A., "Development of a Reconstituted Water Medium and Preliminary Validation of the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)," *Journal of Applied Toxicology*, Vol 7, 1987, pp. 237–244; Courchesne, C. L., and Bantle, J. A., "Analysis of the Activity of DNA, RNA, and Protein Synthesis Inhibitors on *Xenopus* Embryo Development," *Teratogenesis Carcinogenesis and Mutagenesis.*, Vol 5, 1985, pp. 177–193; Dumont, J. N., Schultz, T. W., and Epler, R. G., "The Response of the FETAX Model to Mammalian Teratogens," *Teratology*, Vol 27, 1983, p. 39a; Sabourin, T. D., Faulk, R. T., and Goss, L. B., "The Efficacy of Three Non-mammalian Test Systems in the Identification of Chemical Teratogens," *Journal of Applied Toxicology*, Vol 5, 1985, pp. 225–233; Sabourin, T. D., and Faulk, R. T., "Comparative Evaluation of a Short-term Test for Developmental Effects Using Frog Embryos." In: *Banbury Report 26: Developmental Toxicology: Mechanisms and Risk*, Cold Spring Harbor Laboratory, 1987, pp. 203–223.

(3) Schuler, R., Hardin, B. D., and Niemer, R., "*Drosophila* as a Tool for the Rapid Assessment of Chemicals for Teratogenicity," *Teratogenesis Carcinogenesis and Mutagenesis*, Vol 2, 1982, pp. 293–301; Greenberg, J., "Detection of Teratogens by Differentiating Embryonic Neural Crest Cells in Culture: Evaluation as a Screening System, *Teratogenesis Carcinogenesis and Mutagenesis.*, Vol 2, 1982, pp. 319–323; Kitchin, K. T., Schmid, B. P., and Sanyal, M. K., "A Coupled Microsomal-Activating/Embryo Culture System: Toxicity of Reduced Betanicotinamide Adenine Dinucleotide Phosphate (NADPH)," *Biochemical Pharmacology*, Vol 30, 1981, pp. 985–992.

(4) U.S. Environmental Protection Agency, *Federal Register*, Vol 49, Feb. 7, 1984, pp. 4551–4554.
(5) U.S. Environmental Protection Agency, *Federal Register*, Vol 50, July 29, 1985, pp. 30784–30796.
(6) International Technical Information Institute, *Toxic and Hazardous Chemicals Safety Manual*, Tokyo, Japan, 1977; Sax, N. I., *Dangerous Properties of Industrial Materials*, 5th Ed., Van Nostrand Reinhold Co., New York, NY, 1979; Patty, F. A., ed., *Industrial Hygiene and Toxicology*, Vol II, 2nd Ed., Interscience, New York, NY, 1963; Hamilton, A., and Hardy, H. L., *Industrial Toxicology*, 3rd Ed., Publishing Sciences Group Inc., Acton, MA, 1974; Gosselein, R. E., Hodge, H. C., Smith, R. P., and Gleason, M. N., *Chemical Toxicology of Commercial Products*, 4th Ed., Williams and Wilkins Co., Baltimore, MD, 1976, Green, M. E., and Turk, A., *Safety in Working with Chemicals*, Macmillan, New York, NY, 1978; National Research Council, *Prudent Practices for Handling Hazardous Chemicals in Laboratories*, National Academy Press, Washington, DC, 1981; Walters, D. B., ed., *Safe Handling of Chemical Carcinogens, Mutagens, Teratogens and Highly Toxic Substances*, Ann Arbor Science, Ann Arbor, MI, 1980; Fawcett, H. H., and Woods, W. S., eds., *Safety and Accident Prevention in Chemical Operations*, 2nd Ed., Wiley-Interscience, New York, NY, 1982.
(7) National Council on Radiation Protection and Measurement, "Basic Radiation Protection Criteria," NCRP Report No. 39, Washington, DC, 1971; Shapiro, J., *Radiation Protection*, 2nd Ed., Harvard University Press, Cambridge, MA, 1981.
(8) National Institutes of Health, "NIH Guidelines for the Laboratory Use of Chemical Carcinogens," NIH Publication No. 81-2385, Bethesda, MD, May 1981.
(9) Seegert, G. L., and Brooks, A. S., "Dechlorination of Water for Fish Culture: Comparison of the Activated Carbon, Sulfite Reduction and Photochemical Methods," *Journal of the Fisheries Research Board of Canada*, Vol 35, 1978, pp. 88–92.
(10) Montgomery, J. M., *Water Treatment: Principle and Design*, John Wiley and Sons, Inc., NY, 1985, pp. 330–332.
(11) Davey, E. W., Gentile, J. H., Erickson, S. J., and Betzer, P., "Removal of Trace Metals from Marine Culture Media," *Limnology and Oceanography*, Vol 15, 1970, pp. 486–488.
(12) Bullock, G. L., and Stuckey, H. M., "Ultraviolet Treatment of Water for Destruction of 5 Gram-Negative Bacteria Pathogenic to Fishes," *Journal of the Fisheries Research Board of Canada*, Vol 34, 1977, pp. 1244–1249.
(13) EPA Water Quality Criteria for Water (EPA/440/5-86/001), 1986; Pierce, B. A., "Acid Tolerance in Amphibians," *Bioscience*, Vol 35, 1985, pp. 239–243.
(14) Birge, W. J., and Black, J. A., "*In Situ* Acute/chronic Toxicological Monitoring of Industrial Effluents for the NPDES Biomonitoring Program Using Fish and Amphibian Embryo-larval Stages as Test Organisms," OWEP-82-001. Office of Water Enforcement and Permits, U.S.E.P.A., Washington, DC, 1981, pp. 121.
(15) Fort, D. J., James, B. L., and Bantle, J., "Evaluation of the Developmental Toxicity of Five Compounds with the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) and a Metabolic Activation System." *Journal of Applied Toxicology*, In Press.
(16) Deuchar, E. M., "*Xenopus: The South African Clawed Frog*," Wiley, New York, NY, 1975.
(17) Deuchar, E. M., "*Xenopus laevis* and Developmental Biology," *Biological Reviews*, Vol 47, 1972, pp. 37–112.
(18) Nieuwkoop, P. D., and Faber, J., *Normal Tables of Xenopus laevis* (Daudin), 2nd Ed., North Holland, Amsterdam, 1975.
(19) Horning, W. B., and Weber, C. I., "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms," 1985, (EPA/600/4-89/001, pp. 105–162).
(20) Dawson, D. A., Fort, D. J., Newell, D. L., and Bantle, J. A., "Developmental Toxicity Testing with FETAX: Evaluation of Five Compounds," *Drug and Chemical Toxicology*, Vol 12, 1989, pp. 67–75.
(21) Berg, E. L., (ed.), "Handbook for Sampling and Sample Presentation of Water and Wastewater," EPA 600/4-82-029, National Technical Information Service, Springfield, VA, 1982.
(22) U.S. Environmental Protection Agency, "Methods for Chemical Analysis of Water and Wastes," EPA 600/4-79-020 (Revised March 1983), National Technical Information Service, Springfield, VA, 1983; Strickland, J. D. H., and Parsons, T. R., A Practical Handbook of Seawater Analysis, Bulletin 167, Fisheries Research Board of Canada, Ottawa, 1968; National Handbook of Recommended Methods for Water-Data Acquisition," U.S. Department of Interior, Reston, VA, 1977; American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Standard Methods for the Examination of Water and Wastewater, 16th ed., Washington, DC, 1985.
(23) Birge, W. J., and Black, J. A. In: *Copper in the Environment, Pt II. Health Effects*, Wiley, New York, 1979, pp. 373–399; Birge, W. J., Black, J. A., Westerman, A. G., and Hudson, J. E., In: *The Biogeochemistry of Mercury in the Environment*. Elsevier/North-Holland Biomedical Press, 1979, pp. 629–655.