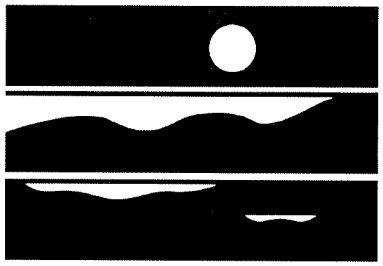

Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) for Soil Toxicity Screening

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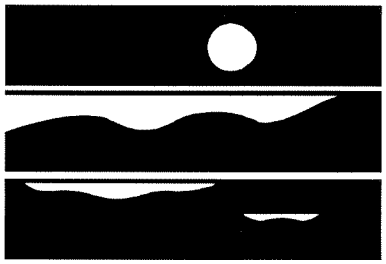

Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) for Soil Toxicity Screening

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Appendices

Summary of Test Conditions for the Frog Embryo Teratogenesis Assay *Xenopus* (FETAX)

Test type:	96-hour static renewal
Temperature:	24±2°C
Photoperiod:	Continuous darkness
Test vessel:	60mm glass petri dishes
Test solution volume:	10ml per dish
Replacement of test solution:	Every 24-hours
Test organism:	Normally cleaving embryos (Stage 8 - 11) of the South African clawed frog (*Xenopus laevis*)
Number of replicates/sample:	3
Number of replicates/control:	4
Number of organisms/replicate:	25
Eluate pH:	6.5 - 9.0, do not adjust if pH outside of range, do not run test
Alkalinity and hardness:	16 - 400mg/L as $CaCO_3$
Endpoints:	Survival and malformation
Positive control:	6-aminonicotinamide in FETAX solution
Negative control:	FETAX solution

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1. Introduction

1.1 Application and Background

The Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) described in this protocol is intended for use in screening level assessments of soil toxicity at hazardous waste sites being investigated under the Washington Model Toxics Control Act (MTCA) Cleanup Regulation. FETAX is a 96-hour static renewal, whole embryo bioassay, that may be used to evaluate the developmental toxicity of a test material. In this protocol, FETAX will be used to assess the toxicity of eluates prepared from field-collected soil samples. Serial dilutions are not required.

FETAX is conducted at the embryonic stage of development. This stage of development has demonstrated sensitivity and may provide information that is useful in estimating the chronic toxicity of a test material (Bantle *et al.*, 1989). FETAX uses dual endpoints, mortality and malformation of embryos to assess developmental toxicity. Validation tests of FETAX have indicated close correlation with other bioassays used for toxicity assessment of single compounds (Bantle *et al.*, 1990; Fort *et al.*, 1989), as well as mixtures (Dawson *et al.*, 1988 and 1989; Bantle *et al.*, 1989; Fort *et al.*, 1995).

The testing procedures described in this document are based on the *Standard Guide for Conducting FETAX* (ASTM, 1991) and the *Atlas of Abnormalities: A Guide for the Performance of FETAX* (Bantle *et al.*, 1990).

1.2 Health and Safety

Testing of materials from hazardous waste sites may involve significant risks to laboratory personnel. All persons possibly exposed to or involved with the material(s) to be tested should protect themselves by taking all necessary safety precautions to prevent physical harm. Procedures designed to prevent inhalation or dermal absorption of test materials should be observed. Each laboratory should be adequately equipped with all necessary safety equipment prior to initiation of toxicity testing.

For guidance on safe practices when conducting toxicity tests, see laboratory and general industrial safety manuals, also Peltier and Weber (1985) and USEPA (1977).

2. Sample Requirements

2.1 Sample Size

Each sample requires approximately 45g of soil to prepare sufficient eluate for testing. This amount of soil yields approximately 180ml of eluate, when using a soil to water ratio of 1:4. Each sample is tested using three replicates (a replicate is defined as one 10ml aliquot of the bulk soil eluate). Renewal/exchange of the eluate occurs every 24 hours during the 96-hour testing period. Consequently, 120ml of eluate (three replicates of 100% eluate: 10ml/day x 4 days= 120 ml) are required for each sample. If chemical characterization of the soil is to be performed in conjunction with the toxicity test, or if concentration-response experiments are to be conducted, additional soil will need to be collected. At the time of sample collection containers should be filled to reduce headspace to a minimum.

2.2 Sample Storage

Upon arrival at the laboratory facility, all soil samples should be stored in a dark, vented refrigerator at 4±2°C. Samples must be properly sealed and packaged when being moved from storage to work spaces as exposure to air may volatilize some substances. When the samples are being prepared for testing, they should be opened and dispensed within a fume hood. Sample preparation should begin as soon as possible, preferably within 24 hours of sample collection. Realizing that it is not always possible to initiate the test within 24 hours of collection, under no circumstances should the samples be held longer than 14 days from collection to test initiation. The toxicity of some samples may be affected when held beyond 14 days due to loss or degradation of contaminants present in the samples. This is especially true for volatile organics. It is recommended that standard Chain-of-Custody procedures be followed when handling and analyzing samples collected from hazardous waste sites.

3. Supplies

3.1 Equipment

The following equipment is required to perform the FETAX test:

- Environmental chamber, incubator, or equivalent facility with ability to maintain temperature at 24±2°C. The test is conducted in darkness.
- Water purification system that produces Type I reagent grade water (see Appendix A for specifications) may be required depending on the quality of source water
- Test vessels, 60mm glass petri dishes with covers.
- Dissecting microscope (30x) for selecting embryos (Stage 8 - 11) for use in FETAX and assessing embryo malformations at test termination.
- Volumetric flasks and graduated cylinders, class A, borosilicate glass or non-toxic plastic labware, 10 to 1000ml, for culture work and preparation of test solutions.
- Volumetric pipettes, class A, 1 to 100ml.
- Serological pipettes, 1 to 10ml, graduated.
- Pasteur pipettes with fire polished edges, 1.5 - 3.0mm ID.
- Pipette bulbs and filters.
- Disposable polyethylene or glass pipettes, droppers, and glass tubing with fire polished edges, 1.5 - 3.0mm ID.
- Wash bottles, for rinsing small glassware, instrument electrodes, and probes.
- Glass or electronic thermometers for measuring water temperatures.
- Bulb thermograph or electronic-chart type thermometers for continuous recording of temperature.

- Dissolved oxygen (D.O.), pH, and specific conductivity meters for routine physical and chemical measurements. Portable, field-grade instruments are acceptable for measurements, unless the test is being conducted to specifically measure the effect of one of the above parameters.

- Rotary tumbler or similar apparatus (end over end mixing) to prepare soil eluates

3.2 Materials

The following materials are required to perform the FETAX test:

Dilution Water

The minimum criteria for acceptable dilution water are listed below:

- Available in adequate supply with uniform quality.
- Will allow satisfactory survival and reproduction of adult *Xenopus* for the duration of acclimation and testing without showing signs of stress, such as lethargy or other unusual behavior.
- Will not adversely affect the results of the test.

Depending on the quality of the laboratory water, a number of options may be considered when preparing dilution water. If the laboratory has high grade tap or well water, only a minor purification system will be necessary such as distillation, deionization or activated carbon filtration. Regardless of the purification process chosen, dilution water quality should, at a minimum, meet the specifications for Type I reagent grade water (ASTM, 1993). Specifications for Type I water are listed in Appendix A. Chlorinated water must not be used as dilution water because residual chlorine might be toxic to aquatic organisms.

Dilution water is considered to be of uniform quality if the monthly ranges of hardness, alkalinity and specific conductance are <10% of their respective averages, and if the monthly range of pH is <0.8 units of its average.

Adult Culture Water

De-chlorinated tap water may be used to culture adult frogs as long as residual chlorine and its oxidants are reduced to levels that do not affect survival and reproduction. Refer to section 4.2 for a complete description of the requirements for adult culture water.

FETAX Solution

FETAX solution is used as the test medium in this protocol. FETAX solution is composed of 625mg NaCl, 96mg $NaHCO_3$, 30mg KCl, 15mg $CaCl_2$, 60mg $CaSO_4$, 2 H_2O and 75mg $MgSO_4$ per liter of dilution water. All chemicals must be of reagent grade quality or higher. The pH of the final solution should be 7.6 - 7.9.

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 demonstrated that their use will not adversely affect either survival or growth of the frog embryos.

pH Buffers

Buffers for pH 4, 7, and 10 are required for standards and calibration checks.

Reagents for Hardness and Alkalinity Tests

See USEPA Method 130.2, 310.1 (USEPA, 1979b).

Membranes and Filling Solutions for D.O. Probe

See USEPA Method 360.1 (USEPA, 1979a).

Specific Conductivity Standards

See USEPA Method 120.1 (USEPA, 1979a).

Reference Toxicant

6-Aminonicotinamide (CAS #329-89-5).

3.3 Test Organism

The FETAX protocol described in this document is designed to be used with embryos of the South African clawed frog *Xenopus laevis* (Daudin). Embryos for each test should be cultured from a single pair of breeding adults. Only normally cleaving embryos should be selected for testing. Embryos that are between midblastula (Stage 8) to early gastrula (Stage 11) must be used to start the test (ASTM, 1991).

4. Adult Husbandry and Culture

4.1 Adult Husbandry Facilities

The facilities for maintaining and breeding *Xenopus* adults include an animal room with a photoperiod of 12-hours light/12-hours darkness. Adults may be housed in large aquaria or in fiberglass or stainless steel raceways at densities of 4 - 6 individuals/1800 cm^2 of water surface area. The sides of the tank should be opaque and at least 30cm high. Water depth should be 7 - 14cm. Water temperature for adults should be 23±3°C. Wire or plastic mesh secured across the top of the aquaria may be necessary to prevent adults from escaping.

4.2 Water For Culturing *Xenopus* Adults

Water for culturing adult *Xenopus* must meet the minimum quality specification listed for dilution water (section 3.2). Baseline values for the following constituents should be determined prior to using the water to culture adults: hardness, alkalinity, conductivity and pH. The pH of the water should be 6.5 - 9.0 (USEPA, 1986; Pierce, 1985), preferably slightly alkaline (7.5 - 8.1). If pH falls outside this range, it should be adjusted accordingly. Lower the pH using hydrochloric acid (HCl) or elevate the pH using sodium hydroxide (NaOH). Alkalinity and hardness should both should be between 16 - 400mg/l as $CaCO_3$.

In addition to baseline monitoring, the following items should be measured at least quarterly: total dissolved solids (TDS), total organic carbon (TOC), organophosphorus pesticides, organochlorine pesticides, polychlorinated biphenyls, chlorinated phenoxy herbicides, ammonia, bromide, beryllium, cadmium, chromium, copper, lead, manganese, mercury, nickel, selenium, silver and zinc. The detection limit for each of the above listed contaminants should be below the lowest concentration that has been shown to adversely affect the frogs in water (ASTM, 1991). Table 1 lists the recommended maximum concentrations for some of the metals listed above.

Table 1. Recommended Maximum Concentrations of Selected Metals in Adult Culture Water.

Metal[1]	Recommended Maximum Concentration *u*g/l
Cadmium	10.0
Lead	5.0
Mercury	0.14
Nickel	25.0
Selenium (unpublished)	140.0
Zinc	70.0

[1]=Tested in FETAX at 100 mg/l hardness as $CaCO_3$. Values reported are one-tenth of the minimum concentration to inhibit growth (ASTM, 1991).

Natural water is preferred for culture of adults. 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 surface water. If surface water is used as a source for culture water, the intake should be positioned to minimize fluctuations in quality while maximizing the concentration of dissolved oxygen. These measures help ensure low concentrations of sulfide and iron. Water temperature should be adjusted to 23±3°C before being used to culture adults.

De-chlorinated water may be used to culture adults as long as residual chlorine and its oxidants are reduced to levels that do not affect survival and reproduction. Because de-chlorination is often incomplete, de-chlorinated water should only be used as a last choice. Sodium bisulfate is probably better for de-chlorinating water than sodium sulfate, and both are more reliable than carbon filters, especially for removing chloramines. Fluorides can be removed by passage over activated alumina columns. In addition to residual chlorine, chloramines and fluoride, municipal drinking water often contains unacceptably high concentrations of copper, lead and zinc. Excessive concentrations of most metals can usually be removed with a chelating resin (ASTM, 1991).

Water for culturing adults may be aerated by air stones or surface aerators. Air used for aeration should be free of fumes, oil and water. Compressed air supplies may be contaminated with oil or water containing rust or sludge. Some compressed air supplies may also have a high level of carbon monoxide. A low pressure blower will provide high quality air, provided the source of air supply is uncontaminated. Adequate aeration will stabilize pH, bring concentrations of D.O. and other gases into equilibrium with ambient conditions, as well as minimize oxygen demand and concentrations of volatile compounds. However, it is not absolutely necessary to aerate the water for culturing *Xenopus* (ASTM, 1991).

Filtration through bag, sand, sock or depth type cartridge filters may be used to keep the concentration of particulate matter low in the adult culture water. This large-particle filtration may serve as a pretreatment before filtration through a finer filter. Organics may be removed by filtration through activated charcoal filters. Charcoal filters should be cleaned on a monthly basis.

4.3 Adult Husbandry Requirements

Adult frogs of breeding age 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. The *Atlas of Abnormalities* should be consulted for examples (Bantle *et al.*, 1990). Care should be taken to insure that only healthy, sexually mature animals are placed in the colony.

Selection

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

Diet

The minimum recommended diet for adults should be three feedings per week of ground adult beef liver that meets USDA standards for human consumption. Liquid multiple vitamins should be added to the ground beef liver (Table 2). Concentration of vitamins from 0.05 to 0.075ml/5g liver are appropriate. All food should be screened for the test material, if the test material is present in the environment.

Table 2. Recommended Concentration of Vitamins[1] added to adult frog food.

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

[1]= per ml of solution.

Temperature

Adults should be housed and maintained at 23±3°C.

Circadian rhythm

Adults should be maintained on a constant photoperiod of 12 hours light/12 hours dark, because the role that circadian rhythm plays in *Xenopus* reproduction has not been investigated.

4.4 FETAX Test Facilities

Facilities for conducting the FETAX bioassay include a controlled-environment room or incubator without light. The incubator must be capable of maintaining a temperature of 24±2°C. Covered 60mm glass petri dishes serve as test vessels. A binocular dissection microscope capable of magnification up to 30x is necessary to count and evaluate abnormal embryos.

Construction materials and commercially purchased equipment that may contact stock solutions, test solutions or any water into which adult frogs or embryos will be placed should not contain any substances that can be leached or dissolved by aqueous solutions. Additionally, materials and equipment that contact stock solutions and test solutions should be chosen to minimize sorption of toxicants from water. Glass, type 316 stainless steel and fluoroplastics must be used whenever

possible to minimize leaching, dissolution and sorption of contaminants. Natural rubber, copper, brass, galvanized metal, and lead must not come in contact with dilution water, stock solutions, or test solutions before or during exposure of frogs (ASTM, 1991).

Before FETAX is conducted in a new testing facility it is advisable to conduct a non-toxic test, in which all test chambers contain FETAX solution/dilution water with no added test material. The embryos should grow, develop, and survive in numbers consistent with an acceptable test (see Section 11). The magnitude of chamber to chamber variation should be evaluated.

5. Culture and Preparation of Test Embryos

5.1 Breeding Facilities

Both 18ℓ and 38ℓ aquaria have been used successfully for breeding *Xenopus*. The aquaria should be fitted with a 1cm mesh (nylon or plastic) screen suspended approximately 3cm from the bottom of the tank to prevent disturbance of deposited eggs. Hardware cloth or other metal mesh must not be used. The aquaria should be fitted with air stones or other bubblers to oxygenate the water (ASTM, 1991). A screen or mesh should be secured (heavy book, duct tape) to the top of the breeding chamber to prevent escape of the breeding pair from the chamber. The screen or mesh should be covered with an opaque porous material such as a black cloth to enhance breeding activity.

5.2 Breeding of Adults for Embryos

The frogs should be bred in the same water in which the test is to be conducted. To induce breeding, the male receives 250 - 500IU of human chorionic gonadotropin (HCG) via subcutaneous injection into the dorsal lymph sac. The reader is referred to the *Atlas of Abnormalities* for illustration of injection sites (Bantle *et al.*, 1990). The female receives 500 - 1000IU of HCG in a similar manner. The amount of HCG injected depends on the time of year and condition of the adults. Lower doses are usually adequate in spring, while higher doses may be required in winter. The hormone concentration should be 1000IU/ml in sterile 0.9% NaCl. A 1ml tuberculin syringe fitted with a 1.2cm long, 26-gauge needle should be used to make the injection. Larger bore needles might allow leakage of hormone from the injection site. After injection, a single pair of adults are placed in each breeding chamber.

Amplexus normally ensues within 2 - 6 hours, and egg deposition follows about 9 - 12 hours after injection. The eggs should be inspected immediately for fertility and quality. The fertility rate should be >75%. Eggs laid in "strings" or not perfectly round will develop abnormally and should not be used.

It is necessary to keep clutches separate for testing because embryos from a particular mating pair may develop poorly, even though they initially appear acceptable. This condition would result in all the embryos being discarded, if embryos from different mating pairs are combined for testing.

5.3 Preparation of Embryos for FETAX

De-jellying of embryos should begin immediately following the end of egg laying. De-jellying of embryos should be carried out by gentle swirling for 1 to 3 minutes in a 2% (w/v) solution of

L-cysteine (CAS # 52-90-4) in FETAX solution. This solution should be adjusted to a pH of 8.1 with 1 N NaOH. The L-cysteine solution is prepared immediately prior to use (ASTM, 1991)

Pour off as much of the cysteine solution as possible. Add FETAX solution to the flask and gently swirl the embryos. Pour off the FETAX solution. Repeat at least three times to rinse the cysteine solution from the embryos and flask.

Before embryos can be assigned to a test vessel, the stage of development and quality of embryo must be determined under a dissecting microscope. Nieuwkoop and Faber (1975) must be used in all staging of embryos. Only normally cleaving embryos are selected for testing. The *Atlas of Abnormalities* should be consulted to determine if the embryos are normal.

Two levels of embryo selection are recommended. 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 (ASTM, 1991). Abnormal pigmentation should be viewed as an indicator of bad embryos. Either Nieuwkoop and Faber (1975) or the *Atlas of Abnormalities* may be used as a reference to determine whether the cleavage pattern is normal. Embryos that are in 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 beyond Stage 11 have commenced organogenesis. The sorting of embryos should be done in 100mm petri dishes under the dissecting microscope. A large bore blood bank Pasteur pipette can be used to transfer embryos at this stage without harm.

After the required number of normally developing embryos have been selected, they are randomly assigned to treatment groups. Each eluate sample should have three replicates, each containing 25 embryos and 10ml of test solution. Each negative control should also have three replicates with 25 embryos. Embryos should be randomly assigned to all test dishes, which should be randomly assigned to their positions in the incubator.

6. Preparation of Soil Samples

6.1 Moisture Fraction Determination

The initial moisture content of the bulk soil samples must be determined to calculate the dry weight of each soil sample.

Place a 25g aliquot of the bulk soil sample in a clean, crystallizing dish and weigh it to obtain the initial wet weight for moisture content calculations. The combined weight of the sample and dish equals the initial wet weight.

Dry the sample at 103 - 105°C for 24 hours. After drying, place the sample into a desiccator to cool. After cooling, weigh the dried sample. The combined weight of the dish and the dried sample equals the final dry weight.

Calculate the moisture fraction of the sample using the following formula:

1) **MF= (I - F)/[A - (I - F)] * 100**

where MF= Moisture fraction of bulk soil (in %)
I= Initial wet weight of sample + crucible (in g)
F= Final dry weight of sample + crucible (in g)
A= Initial aliquot weight (in g)

Example Calculation:

I= 40g
F= 35g
A= 25g

MF= (40g - 35g)/[25g - (40g - 35g)] * 100= 25%

6.2 Eluate Preparation

The following procedure is used to prepare the soil eluates. Dilution water should be used to prepare all eluates from soil samples.

1. Add a weight of dilution water equal to 4 times the dry weight of the soil sample.

2. Shake for 48-hours (30±2rpm) at constant temperature (22±2°C) in darkness. Use a rotary tumbler (end-over-end mixing) or similar apparatus. Allow zero head space, if possible, in the mixing containers to prevent loss of volatile substances.

3. Allow to settle overnight in refrigerated (4±2°C) storage, decant and record pH. Place the eluate in a refrigerated centrifuge (4°C) for about 20 minutes at 8,000rpm (5500 - 6000x g-force) or until supernatant is clear. Do not filter eluate because filtering may remove particulate material that may have toxicants adsorbed onto it, resulting in an underestimation of toxicity.

4. Add the ingredients to the eluate to make FETAX solution (see Section 3.2). Allow to settle overnight in refrigerated (4±2°C) storage.

5. Store enough eluate for the first 24-hour testing period in a vented refrigerator (4±2°C) until needed. This solution should be used within 24 hours. The temperature of the eluate must be equilibrated to 24±2°C before adding embryos.

6. Divide the remaining eluate samples into aliquots of 10ml with no head space. Either refrigerate (4±2°C) the aliquots with no headspace or freeze (-20 to - 80°C) the aliquots until needed for the daily test renewal.

7. Preparation of Reference Toxicant (6-Aminonicotinamide)

Reference toxicant testing must be conducted, at a minimum, on a monthly basis. The test may be conducted either separately or concurrently with a FETAX test. However, it is recommended that acceptable response of the test organisms be established prior to testing samples.

FETAX solution should be used in preparation of the reference toxicant solution. Commercial sources for 6-aminonicotinamide (CAS # 329-89-5) should specify the physiochemical properties and purity of the compound. The purity should be >99%. The 96-hour median lethal concentration (LC50) for 6-aminonicotinamide is 2500mg/l (95% CI= 2350 - 2650), and the 96-hour median effect concentration (EC50) for malformation is 5.5mg/l (95% CI= 3.9 - 6.9), or a Teratogenic Index (TI) of 455 (Dawson *et al.*, 1989). Each positive control consists of four dishes of 25 embryos each. Embryos for the LC50 determination are exposed to 2500mg 6-aminonicotinamide/ℓ and embryos for the EC50 are exposed to 5.5mg 6-aminonicotinamide/ℓ. The mortality (LC50) and malformation (EC50) observed should be between 10 - 90%. For example, at 2500 mg/l exposure, 10 - 90 of the 100 embryos should die by 96 hours. Only those biological responses related to mortality and malformation are considered in this test evaluation.

Reference Toxicant Data Analysis: Report embryos that died or appeared affected in the reference toxicant treatment and the corresponding concentration of the reference toxicant. Calculate the 96-hour LC50, 96-hour EC50 and the 95% confidence limits for the positive controls and check the results graphically. If the Trimmed Spearman-Karber method is used, follow the methods described by Hamilton *et al.* (1977). If the Probit technique is used, follow the methods described by Weber *et al.* (1989). Other statistical method(s) may be substituted for the trimmed Spearman-Karber or Probit techniques provided that they have comparable power.

8. Conducting the Test

8.1 Preparation of Test Petri Dishes

Mark each petri dish lid or bottom with the appropriate sample/replicate number and any other necessary information. Use a minimum of three replicates per sample. Temperature of the sample should be equilibrated to 24±2°C just prior to initiating the test and maintained at that temperature throughout the testing period. Test temperatures higher than 26°C result in malformation or mortality. Low temperatures inhibit growth of the controls, preventing them from reaching stage 46 in 96 hours.

Place a 10ml aliquot of the 100% eluate into each petri dish. Measure the pH, conductivity, alkalinity, and hardness of the eluate in one replicate of each sample and one replicate of each control. All water quality measurements should be within the specifications listed for the adult culture water. After completing the water quality measurements the dishes should be covered until needed.

8.2 Negative Control

Preparation of the negative control involves placing 10ml of FETAX solution into a individual petri dish. A minimum of four replicates are used for the negative control. FETAX solution is prepared 24 - 72 hours before use and may be stored at room temperature. No toxicant is added to the FETAX solution. The FETAX solution in the negative controls is exchanged every 24 hours.

9. Test Monitoring

At test termination (96 hours), at least 90% of the negative controls must achieve Stage 46 of development for test acceptability (ASTM, 1991). Deviations from this standard time of exposure must be reported.

9.1 Eluate Measurements and Solution Renewal

Eluate Measurements

The D.O., pH, conductivity, alkalinity, and hardness should be measured at the beginning and end of the test period in one replicate of each eluate sample and one replicate of each control. All measurements should be recorded on the data collection cover sheet.

Test Solution Renewal

The test renewal procedure involves replacement of eluate every 24 hours during the testing period. All aliquots must be removed from refrigeration or freezer, if applicable, and thawed at ambient temperature to achieve 24±2°C prior to test renewal exchange. Measure the pH of one replicate of each control and each test sample before renewing the test solution. Renewal should be accomplished by removing the old test solution with a Pasteur pipette and appropriately discarding it. Fresh test/control solution should then be added to each petri dish. Use a clean pipette for each test sample and each control. The orifice of the Pasteur pipette should be enlarged and fire polished to accommodate embryos without damage in case they are accidentally picked up. The renewal exchange should proceed quickly to minimize embryo desiccation.

9.2 Embryo Observations

Survival

Observations are made every 24±2 hours under a dissecting microscope. All dead embryos must be tallied, removed and discarded each 24 hours. If dead embryos are not removed, microbial growth can occur that might attack and kill other live embryos. Death at 24 hours (Stage 27) is defined as alteration of the skin pigmentation, diminishing structural integrity, and lack of irritability. Death is defined as lack of heartbeat at 48 hours (Stage 35), 72 hours (Stage 42) and 96 hours (Stage 46). At 96 hours of exposure (Stage 46 of negative controls), the number of live embryos is recorded. All dead embryos are removed and discarded. Surviving embryos are deeply anesthetized with 2% (w/v) methyl succinic acid diethyl ester (M.S. 222) and preserved in 3% formalin.

10. Data Analysis and Reporting

10.1 Endpoint Data on Embryos

Survival

See Section 9.2 above for definitions of endpoints.

Malformation

Preserved embryos are evaluated under the dissecting microscope and the following observations are recorded: Malformations must be recorded at the end of 96 hours. *The Atlas of Abnormalities* should be used in scoring malformations. This is especially true for 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 inter-laboratory comparison (see Appendix D for example format). Calculate the percent malformation for each replicate at the end of the 96-hour period and enter the result on the data collection sheet. Also report the average malformation for the sample across replicates.

10.2 Supplemental Data Observations on Embryos

Different types of data (locomotion and hatchability) have been collected in FETAX and may be included in addition to the survival data listed above. In the case of locomotion, scoring is subjective. Locomotion observations must be performed prior to anesthetizing the surviving embryos at test termination, while hatchability must be counted and recorded at the end of the first 48 hours during the test procedure. Record on the data sheet any sublethal effects that were observed.

Hatchability (optional)

The embryos hatch from the fertilization membrane between 18 and 30 hours. The number failing to hatch at 48 hours 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-hour test length, except that it is much easier to score hatching.

Locomotion (optional)

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.

10.3 Data Report

The following is a suggested format for recording relevant information about the test (see Appendix B, C, and D for examples). Other formats are acceptable for reporting purposes, provided the same information is included.

The cover sheet should contain specific information regarding the test including the following:

- Name of the site where the soil was collected and other identifying information.
- Date and time the test was started.
- Name of the person performing the test.
- Conductivity, D.O., pH and hardness of the eluate.
- Any problems/deviations from the protocol.

The laboratory test report should include the data collection and observation sheets. If not provided elsewhere, also include the following information:

Data Handling

Calculate the percent survival for each replicate of the test and control samples at the end of the 96-hour period and enter the results on the data collection sheet. Also report the average survival for each test sample and each control across replicates.

Description of Adverse Effect

Report any malformation in the embryos (see Appendix D) and a summary of supplemental observations of other effects or symptoms noted.

Statistical Analysis

List or describe all statistical procedures or/and software used for data analysis. Use Dunnett's Test (Weber *et al.*, 1989; Eirkson *et al.*, 1987; Zar, 1984) or other method with comparable power.

Control Charts

Prepare a control chart for the positive control series by plotting successive toxicity values (LC50) and examining the results to determine whether they are within the prescribed limits. Recalculate the mean and upper and lower control limits (±25%) with each successive point until the statistics stabilize. Maintain a running plot for the toxicity values of successive tests with the reference toxicant. For further details, see Weber *et al.* (1989) or Greene *et al.* (1988).

Test Organisms

Detailed information about the adult frogs -- including scientific name, weight, source, age, life stage, observed diseases and treatments, culture methods, quality and quantity of food fed to the colony, and acclimation procedures used -- should be recorded.

Test Conditions

Provide a description of the test conditions, especially if there was a deviation from this document in photoperiod, pH, temperature, the number of replicates per treatment, and number of organisms per replicate. Report the method of randomly assigning organisms and distributing test containers.

Test Containers

Report the size, volume and amount of eluate solution in each container, number of test containers per sample and preparation of the test containers.

Chemical Analyses

Report the results of all chemical analyses of soil samples and eluate samples, including methods, method validations and reagent blanks.

Dilution Water

Report the source of the dilution water, the date and time of its collection and a description of any pretreatment. Include the most recent quarterly analysis of adult culture water.

Reporting of Protocol Deviations/Problems

Any deviation from the direction provided in this document and anything unusual about the test (e.g. equipment failure, fluctuations in temperature or other environmental conditions, organism disease) must also be included in data reports.

11. Quality Assurance/Quality Control (QA/QC)

11.1 Requirements and Specifications

Quality assurance (QA) practices for toxicity testing consist of all aspects that could affect data quality including: 1) sample collection and handling, 2) source and condition of the test organisms, 3) condition of equipment, 4) test conditions, 5) instrument calibration, 6) replication, 7) use of reference toxicant, 8) record keeping, and 9) data evaluation. The QA guidelines presented here are adapted from Green *et al.* (1988). For general guidance on good laboratory practices related to toxicity testing see FDA (1978), USEPA (1979b, 1980a and 1980b), and DeWoskin (1984).

Soil Sampling and Handling

Soil samples collected for testing must be handled and stored as described in Section 1.

Facilities, Equipment and Test Chambers

Laboratory temperature control equipment must be adequate to maintain the required temperature (24±2°C) throughout the test.

Analytical Methods

Routine chemical and physical analyses must include established QA practices (USEPA 1979b,c).

Calibration and Standardization

Instruments used for routine chemical and physical parameters -- such as pH, D.O., temperature, conductivity, alkalinity, and hardness -- must be calibrated and standardized according to instrument manufacturers protocols.

Test Conditions

Eluate and air temperatures must be maintained within the limits specified for the test. The temperature and pH should be measured at the beginning of each 24±2-hour exposure period in one replicate of each eluate sample and one replicate of the controls. The D.O., pH, conductivity, alkalinity and hardness of the eluate must be checked at the beginning and end of the test period.

Dilution Water

The dilution water used in the bioassay must be of high quality or treated according to the specifications in Sections 3.2 and 4.2.

Quality of Test Organisms

All embryos in a test must be of acceptable quality. *Xenopus* adults should be carefully observed daily during holding and acclimation for signs of disease, stress, physical damage, and mortality. The animals should be observed for skin lesions or red patches on the ventral surfaces. Skin lesions are indicative of nematode infection while the red patches indicate *Aeromonas* infection. If the adult frogs show any signs of disease or stress, appropriate action should be taken (see *Atlas of Abnormalities: A Guide for the Performance of FETAX*).

Test Acceptability

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

- Hardware cloth or metal mesh was used as a floor support in the breeding aquarium.
- In the negative controls, if either the mean survival is <90% or the mean malformation occurrence was >7%.
- If 90% of the negative controls did not attain Stage 46 by the end of 96 hours. Low incubator temperature is the most common cause of negative control embryos not attaining Stage 46. If dilution water, rather than FETAX solution, was used in the negative control test, it may not allow embryonic growth at the same rate as FETAX solution.
- The dilution water did not conform to Type I ASTM standards.
- A dilution water or FETAX solution control was not included in the test.
- Staging of embryos was performed using a reference other than Nieuwkoop and Faber (1975).
- The test was conducted using embryos either earlier than Stage 8 (blastulae) or later than Stage 11 (gastrulae).
- All petri dishes were not physically identical throughout the test.
- Petri dishes were not randomly assigned to their positions in the incubator.
- The embryos were not randomly assigned to their petri dishes.
- Required data concerning survival and malformation were not collected.
- The pH of the FETAX solution was <6.5 or >9.0 in the control.
- Dead embryos were not removed after each 24±2-hour intervals.
- If the reference toxicant group had <10% or >90% mortalities of the total 100 embryos in the 2500 mg 6-aminonicotinamide/l (LC50) positive control and/or <10% or >90% malformations of the total 100 embryos at 5.5 mg 6-aminonicotinamide/l (EC50).

Precision

The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with the reference toxicant before they attempt to measure toxicity of soils. The single laboratory precision of each type of test to be used in a laboratory should be determined by performing five or more tests with a reference toxicant. Precision can be described by the mean, standard deviation, and coefficient of variation (CV) of the calculated endpoints of the replicated tests.

Test Sensitivity and Replication

Test sensitivity (precision at low concentration) depends in part on the number of replicates, the probability level selected, and the type of statistical analysis conducted. Three replicates per sample are required in this toxicity test.

Control Charts

Prepare a control chart for the reference toxicant by plotting successive toxicity values (LC50) and examining the results to determine whether they are within the prescribed limits. Recalculate the mean and upper and lower control limits (±25) with each successive point. Maintain a running plot for the toxicity values of successive tests with the reference toxicant. For further details, see Weber *et al.* (1989) or Greene *et al.* (1988).

If the LC50 from a given test with the reference toxicant does not fall in the expected range for the *Xenopus* embryos when using FETAX solution, then the sensitivity of the organisms and the overall credibility of the test system might be suspect. In this case, the source of the difference and the influence on test results should be considered.

Record Keeping

Proper record keeping is mandatory. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt and other pertinent information relating to their history and condition. Additionally, information on the calibration of equipment and instruments, test conditions employed, and the test results must be recorded. Annotations should be kept current to prevent loss of information.

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Appendices

Appendix A: Specifications* for Type I Reagent Grade Water

Parameter	Specification
Total Matter, maximum (mg/l)	0.1
Specific Conductivity, maximum (umhos/cm @ 25°C)	0.06
Electrical Resistivity, minimum (megohm-cm @ 25°C)	16.67
Minimum color retention of potassium permanganate (minutes)	60
Maximum soluble silica	ND
Microbiological classification	A
Organic contaminants	B

*= ASTM, 1983

ND= Not detectable

A= When bacteria levels need to be controlled, reagent grade types should be further classified as follows: Max total bacteria count- A= 0/ml; B= 10/ml; and C= 100/ml

B= Type I water is intended to be used where maximum accuracy and precision is indicated, provided dissolved organic matter is not a possible interference.

Appendix B: Laboratory Data Collection Cover Sheet

Name of Field Site: _____
Date of Field Sample Collection: _____
Chemical Analysis Results of Soil Sample: _____
Identified Contaminants: _____
_____
_____
Suspected Contaminants: _____
_____
_____
Laboratory Test Start: Date_____Time_____
Laboratory Test End: Date_____Time_____
Name of Laboratory Analyst conducting test:_____
Measurements of Eluate:

Parameter	Test Initiation		Test Termination	
	Control	Test Rep	Control	Test Rep
pH				
Conductivity (umhos/cm)				
Alkalinity (mg/l as $CaCO_3$)				
Hardness (mg/l as $CaCO_3$)				
Dissolved Oxygen (mg/l)				

Deviations/Problems: List and describe below.
_____
_____
_____

Appendix C: Laboratory Test Monitoring Data Sheet- FETAX Survival Data

Laboratory Analyst	Lab Test Start Date and Time
Study Site Name/I.D	Lab Test End Date and Time

Sample No./I.D.	Replicate Number	Location in Incubator	Initial pH	24-h pH/No. live	48-h pH/No. live	72-h pH/No. live	96-h pH/No. live	Total Live	Live as Percent of total

Measure and record pH in one replicate per sample.

Comments:_____

Appendix D: Malformation Data Collection Sheet (from ASTM, 1991)

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)																									