

13.0 FETAX TEST METHOD PROTOCOL

13.1 Standard Detailed Protocol

ASTM published a comprehensive guideline for FETAX in 1991 (**Appendix 10**); a revised guideline was published in 1998 (**Appendix 11**). The procedures presented in the ASTM FETAX Guideline (1991, 1998) are considered to be applicable, with modification, to the use of FETAX for conducting tests on the effects of surface and ground waters, solid phase samples such as soils and sediments, and whole bulk soils and sediments. Specific modifications relevant to the testing of water/soil/sediment samples (ASTM, 1991; 1998) are described briefly in the following subsections. Other aspects of the assay are described in **Section 2.1.1**.

13.1.1 Materials, Equipment, and Supplies

Information on materials, equipment, and supplies needed to support the standard FETAX assay, as described in the ASTM FETAX Guideline (1991, 1998), are presented in **Section 2.1.1** of this BRD.

13.1.2 Detailed Procedures for FETAX

General information on procedures for FETAX, including criteria for assay acceptance, as described in the ASTM FETAX Guideline (1991, 1998), are discussed in **Section 2.1.2** of this BRD. For water/soil/sediment samples, information on the identities and concentrations of major ingredients and major impurities, solubility and stability in water, the estimated toxicity to humans, and recommended safe-handling procedures will generally be lacking. However, at a minimum, the pH, hardness, alkalinity, and conductivity of such samples should be measured (ASTM, 1998).

The 1998 ASTM Guideline outlines procedures for solid phase sample testing (ASTM, 1998). Approximately one kilogram of soil or sediment should be collected and expediently sent to the laboratory to minimize holding time. Prior to testing, soil or sediment subsamples should be

thoroughly homogenized. Subsamples are collected with a non-reactive sampling device and placed in a non-reactive storage container. Subsamples are mixed and stirred until texture and color are uniform. The samples are then stored at 4°C until FETAX testing is initiated. It is recommended that samples be tested within two weeks of receipt unless specific circumstances delay testing

FETAX studies should be performed with the following modification for whole soil or sediment testing. Testing may be performed in 250 mL specimen bottles or similar capped vessels equipped with a 55 mL glass tube with Teflon mesh insert as the exposure chamber. For screening tests, 35 g of sediment (dry weight) should be placed in the bottom of the vessel, with the Teflon mesh insert added, and should be filled with 140 mL of FETAX Solution. It is essential that the dilution soil be non-toxic and as chemically and physically similar to the test soil as possible. Care must be taken in interpreting results of soil/sediment dilution experiments in that toxicity results may be altered because of the nature of the soil/sediment used for dilution. The sample must be equilibrated. The top edge of the glass tube must be higher than the water level to prevent larvae from swimming out after day two. This represents four parts of dilution water to one part of soil or sediment. Blastulae stage embryos are placed directly on the mesh insert that rests directly over the top of the soil or sediment in the sediment/water interface region. The test consists of 25 embryos placed in each of four replicates (total of 100 embryos exposed to FETAX Solution), a minimum of 25 embryos exposed to blasting sand (artificial sediment) in each of three replicates (minimum of 50 embryos total), and 25 embryos exposed to the soil or sediment sample in each of three replicates (minimum of 50 embryos total). Blasting or beach sand should be extensively tested beforehand to ensure that it produced less than 10% mortality or malformations after 96 hours. There should also be a reference soil/sediment tested that is non-toxic but represents the soil/sediment characteristics of the site. Dilutions of the soil or sediment should be prepared by mixing the sample with uncontaminated site soil or laboratory reference soil. Four to six dilutions ranging from 0 to 100% soil sample and a FETAX Solution control are typically tested. Each sample should be tested in triplicate. Solutions and soils or sediments should be changed every 24 hours of the four-day test by moving the insert containing the embryos to a fresh jar of diluent water and soil/sediment sample. Dead embryos are removed at this time. Dissolved oxygen and pH should be measured prior to renewal and in waste

solutions from each successive day. Dissolved oxygen, pH, conductivity, hardness, alkalinity, ammonia-nitrogen, and residual chlorine should be measured on separate aliquots of the batches of FETAX Solution used during the study. The measurements must be conducted after the conclusion of the exposure period and oxygen content must be greater than 5.5 mg/L.

13.1.3 Dose-Selection Procedures—Screening Test

Screening tests (control and 100% sample) may be performed prior to multi-concentration definitive testing (ASTM, 1998). Determinations of LC₅₀ and EC₅₀ values are not possible with this approach and responses may be reported as a percent effect.

13.1.4 Endpoints Measured

The same three endpoints of mortality, malformations, and embryonic growth, as described in **Section 2.1.4** of this BRD are applicable to water/soil/sediment studies using FETAX.

13.1.5 Duration of Exposure

Information on appropriate exposure duration for FETAX, as described in the ASTM FETAX Guideline (1991, 1998), are presented in **Section 2.1.5** of this BRD.

13.1.6 Known Limits of Use

With appropriate modifications, FETAX can be used to conduct tests on aqueous effluents; surface and ground waters; leachates; aqueous extracts of water-insoluble materials; and solid-phase samples, such as soils and sediments, particulate matter, sediment, and whole bulk soils and sediments. The test method is incompatible with substances that alter the pH, hardness, alkalinity, and conductivity of the FETAX solution beyond the acceptable range specified by the ASTM FETAX Guideline (1991, 1998).

13.1.7 Nature of the Responses Assessed

Relevant information is provided in **Section 2.1.7** of this BRD.

13.1.8 Appropriate Vehicle, Negative, and Positive Controls

Relevant information on vehicle, negative, and positive controls are provided in **Section 2.1.8** of this BRD. In addition, blasting or beach sand (artificial sediment), extensively tested beforehand to ensure that it produced less than 10% mortality or malformations after 96 hours, should be included as one of the negative controls. There should also be a reference soil/sediment tested that is non-toxic but represents the soil/sediment characteristics of the site. Historically, FETAX studies using water/soil/sediment samples have not employed metabolic activation. Information on the use of 6-AN as a reference compound for FETAX is provided in **Section 2.1.8**. The need and/or usefulness of incorporating metabolic activation into FETAX studies with environmental samples has not been explored.

13.1.9 Acceptable Range of Negative and Positive Control Responses

Relevant information on the acceptable range of vehicle, negative, and positive control response are provided in **Section 2.1.9** of this BRD.

13.1.10 Data Collection

Data collection, as described in the ASTM FETAX Guideline (1991, 1999), is reviewed in **Section 2.1.10** of this BRD.

13.1.11 Data Storage Media

Information on data storage media is described in **Section 2.1.11** of this BRD.

13.1.12 Measures of Variability

Information on measures of variability, as described in the ASTM FETAX Guideline (1991, 1999), is described in **Section 2.1.12** of this BRD. However, formal evaluations of intra- and inter-laboratory variation in FETAX connected with its application to environmental samples have not been published. Also, no measures of variability for historical negative and positive control data were located.

13.1.13 Statistical and Non-Statistical Methods

Information on statistical and non-statistical methods for analyzing FETAX data are discussed in **Section 2.1.13** of this BRD. In a number of FETAX ecotoxicological studies, however, the magnitude of the response, as measured by the incidence of malformations only, has been used rather than a TI value or a MCIG/LC₅₀ ratio, to assess relative developmental hazard (see **Section 16**). The decision criteria described for these studies are often based on non-statistical methods. For screening tests, statistical evaluation of differences in responses between the control and the single-treated group may be evaluated using parametric or non-parametric hypothesis tests for the mortality and malformation responses, and a grouped student's-test for the growth data (p-value <0.05 for all tests).

13.1.14 Decision Criteria

The decision criteria for FETAX, as described in the ASTM FETAX Guideline (1991, 1998), are described in **Section 2.1.4** of this BRD. However, as indicated in **Section 13.1.13**, these decision criteria are seldom used in studies involving environmental samples. Rather, relative activity based on the incidence of malformations seems to be the most common approach for evaluating the results of such studies.

13.1.15 Test Report Information

The information that should be included in the test report for an acceptable FETAX study, as described in the ASTM FETAX Guideline (1991, 1998), is summarized in **Section 2.1.15** of this BRD. Items specific to defined substances need not be considered. Information on dissolved oxygen, pH, conductivity, hardness, alkalinity, ammonia-nitrogen, and residual chlorine measured on separate aliquots of the batches of FETAX Solution used during the study should be provided. Additionally, specifics on sample moisture fraction determination and extract preparation, if conducted, are required.

13.2 Commonly Used Variations in the FETAX Standard Protocol and Rationale

13.2.1 Use of Alternative Species

Although FETAX was designed expressly for the use of *X. laevis*, it might be appropriate to use an endemic species when required by regulations or other considerations. Users are cautioned that many naturally occurring species of frogs are threatened by pollution and habitat loss and the user should carefully consider the ecotoxicological consequences of large-scale collection of local anuran species. Deviations from standard procedures must be reported. The ASTM FETAX Guideline (1991, 1998) states that it will be difficult to compare data from FETAX with data obtained using an alternative species. However, the sensitivity of *Rana pipiens* and *X. laevis* to several developmental toxicants may be quite similar (D. Fort, personal communication). Members of the family Ranidae (e.g., *R. pipiens*) and Bufonidae (e.g., *Bufo fowleri*) might be best suited for FETAX, because the number of eggs or the seasonal availability, or both, are less limited than for other species. Seasonal availability can be extended by two to three months using human chorionic gonadotropin injection. *R. catesbiana* and *B. americanus* are as well suited as *R. pipiens* and *B. fowleri*. 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 (Birge and Black, 1979; Birge et al., 1979).

The ASTM FETAX Guideline (1991, 1998) suggested that reported differences in sensitivity to inorganic mercury should be taken into account when comparing data among amphibian species.

13.2.2 Additional Data and Alternative Exposure Protocols

Other types of data that can be collected in FETAX and alternative exposure protocols are discussed in **Section 2.3** of this BRD.

13.3 Basis for Selection of FETAX

The basis for selection of FETAX is discussed in **Sections 2.3** and **12.1.2** of this BRD.

13.4 Confidentiality of Information

Original data was not sought by NICEATM for any publication involved with the application of FETAX to the identification of developmental hazards in water/soil/sediment samples.

13.5 Basis for FETAX Decision Criteria

See **Section 2.5** for a discussion of the standard FETAX decision criteria. However, as indicated in **Section 13.1.13**, these decision criteria are seldom used in studies involving environmental samples. Rather, relative activity based on the incidence of malformations, seems to be the most common approach for evaluating the results of such studies. Relative activity was used because the studies evaluated were used generally to prioritize sites by relative importance for further investigation and/or remediation.

13.6 Basis for Numbers of Replicates and Repeat Tests in FETAX

In contrast to the ASTM FETAX assay (1991, 1998), most studies with environmental samples have used two, rather than three, definitive tests to define a FETAX study. The relative merit of two versus three replicate definitive tests has not been determined. Each definitive test is

conducted using embryos from a different male/female pair of *X. laevis*. Each test consists of several different concentrations of the test substance with two replicate dishes at each test concentration and four replicate dishes for each control. Each dish contains 20 or 25 embryos. The number of embryos per dish, the number of replicate dishes per sample dilution, and the number of replicate tests per study have not been based on a formal scientific analysis.

13.7 Validation Study Based Modifications to the Standard Protocol

Published information on FETAX validation studies conducted using environmental samples was not located. Modifications to the standard FETAX protocol arising from validation studies employing defined chemicals are described in **Section 2.7** of this BRD.

13.8 Section 13 Conclusions

The 1991 and the revised and expanded 1998 FETAX Guideline published by ASTM are detailed, comprehensive, and well structured. Adequate information is provided on the necessary materials, equipment, and supplies; screening and definitive tests; endpoint (mortality, malformations, and embryonic growth) assessment; nature of the responses assessed; the duration of exposure; data collection and data storage media; measures of variability; statistical and non-statistical methods; test report information; commonly used protocol variations and rationale; the use of alternative species; and the basis for selection of FETAX.

Known limits of use for FETAX with water/soil/sediment samples were not described, except it was stated that the test method is incompatible with environmental samples that alter the pH, hardness, alkalinity, and conductivity of the FETAX Solution beyond the acceptable range specified by the ASTM FETAX Guideline (1991, 1998).

The three decision criteria used to distinguish between a teratogen and a non-teratogen in FETAX were well described in the ASTM FETAX Guideline (1991, 1998). However, as discussed in **Section 2.8** of this BRD, additional effort to evaluate and optimize the standard FETAX decision criteria appears to be warranted.

Selection of the number of embryos per dish (i.e., 20 or 25), the number of replicate dishes per test concentration (i.e., two), and the number of replicate tests per FETAX study (i.e., three) were based on the best scientific judgement of the developers/users of the assay at the time the ASTM FETAX Guideline (1991, 1998) was developed. However, FETAX studies with water/soil/sediments samples have been published based on the use of two replicate definitive tests only (see **Section 16**). A formal analysis of the relative power of FETAX based on two versus three identical definitive tests would be useful.