16.0 FETAX TEST METHOD DATA AND RESULTS

16.1 Availability of a Detailed FETAX Protocol

As described in **Section 2** of this BRD, a comprehensive ASTM Guideline for FETAX was published in 1991 and a revised guideline suitable for testing environmental samples was published in 1998 (ASTM 1991; 1998). The 1998 ASTM FETAX Guideline is provided in **Appendix 11**.

16.2 Availability of Original and Derived FETAX Data

No attempt was made to obtain original data for any environmental sample study considered in this BRD.

16.3 Statistical and Non-Statistical Approaches used to Evaluate FETAX Data

The statistical and non-statistical methods used by the individual investigator to analyze FETAX data obtained are described in **Section 2.1.13**. In a number of environmental sample 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/LC_{50} ratio to assess relative hazard. The decision criteria described for these studies is also not based on 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 t-test for the growth data.

16.4 FETAX Test Results for Individual Water/Soil/Sediment Samples Evaluated to Assess Developmental Toxicity

FETAX test data from 10 publications involving 124 samples were located, reviewed, extracted, and entered into the NICEATM Environmental Sample Database (**Appendix 8**). All 124 samples had been tested using FETAX without metabolic activation; no environmental sample was tested also with metabolic activation. All environmental samples were tested only once. Qualitative data on malformations observed in *X. laevis* were available for 36 chemicals (29%); quantitative malformation data were not provided for any study.

16.4.1 FETAX Testing with Water/Sediment Samples

In an early environmental study conducted by Dawson et al. (1985), discharges from abandoned lead and zinc mines were evaluated for their ability to induce malformations in FETAX. The discharges were characterized as having high concentrations of zinc, iron, and other metals, in addition to having a low pH and low oxygen content. Typical kinds of malformations induced included gut coiling, pericardial and ventral fin edema, microopthalmia, and tail kinking. The authors concluded that the observed effects were likely due to the observed alterations in oxygen, metal content, and pH.

In a related study, Dawson et al. (1988) evaluated the effects of extracts of metal-contaminated sediments and a reference metal toxicant (zinc sulfate) on the development of exposed *P. promelas* and *X. laevis*, using a standard FETAX protocol. Sediments from two contaminated stream sites were extracted with reconstituted culture water at various pH values for 24 hours, and evaluated for developmental toxicity. Developing *P. promelas* and *X. laevis* embryos were exposed for six and four days, respectively. The endpoints assessed were the EC_{50}, LC_{50}, TI, and MCIG. The investigators concluded that zinc was the major developmental toxicant in the sediment extracts, malformations were a more sensitive endpoint that growth inhibition, the pH used during extraction affected the toxicity of the extracted sample, and *P. promelas* was slightly more sensitive than *X. laevis.*

Contaminated groundwater is potentially hazardous to wildlife and humans (Bruner et al., 1998). Using FETAX, ground and surface water samples collected near a closed municipal landfill south of Norman, Oklahoma, demonstrated elevated developmental toxicity risk. More than 35 volatile and 40 semi-volatile and non-volatile compounds were identified in these samples. Many of the contaminants were known xenobiotics and carcinogens. Toxicity was significantly correlated with cumulative rainfall and relative humidity during the three days prior to sampling, but was negatively correlated with the weather conditions for days four to seven preceding sampling. Mortality was positively correlated with solar radiation and net radiation. The results suggest that solar radiation is low during rain events when toxicants are being diluted; however, toxicants are concentrated during periods of high solar radiation as evaporation increases.

Zaga et al. (1998) investigated the possible interaction between ultraviolet radiation and toxicants by examining the effects of exposure to a carbamate pesticide, carbaryl, and ultraviolet radiation on *X. laevis* embryos. The toxicity of 7.5 mg/L carbaryl increased by 10-fold in the presence of ultraviolet-B radiation, indicating photoenhancement of the toxicity of carbaryl. In another study, La Clair et al. (1998) found that a common insect growth regulator, S-methoprene, can react with sunlight, water, and microorganisms and disrupt the normal development of *X. laevis*. When embryos were exposed to S-methoprene degradates, malformations including eye defects and neural tube defects were observed.

Another environmental concern is the direct discharges from industries and municipal wastewater treatment plants. These wastewaters are complex mixtures that contain organic and inorganic compounds. Ciccotelli et al. (1998) investigated the biochemical alterations, such as glutathione-S-transferase (GST) activity, in *X. laevis* caused by exposure to various concentrations of wastewater from a treatment plant. Results of the investigation support the use of *X. laevis* in measuring biochemical alterations that serve as early indicators of environmental hazards. Vismara et al. (1993) used FETAX to evaluate a water purification system by testing the input and output waters from a chemical company for the presence of toxicants. Under the conditions of the test, the percentage of dead embryos following exposure to input water (i.e., untreated wastewater) was 100%, whereas the percentage of dead embryos following exposure to output water(i.e., treated wastewater) was 6.7%.

Malformations and abnormalities have been observed in various species of frogs inhabiting bodies of water throughout the United States. Malformations identified include missing and partial hind limbs, missing or misplaced eyes, microencephaly, ectromelia, ectrodactyly, and internal abnormalities (Fort et al., 1999a, b). A number of factors have been proposed as potential contributors to malformations in natural amphibian populations. These include the presence of developmental toxicants, ionic imbalances, nutritional deficiencies, mineral depletion (e.g., calcium and magnesium), disease (e.g., parasite infestation), UV radiation, and weather conditions (e.g., air temperature, humidity, rainfall) (Bruner et al., 1998; Fort et al., 1999a; Burkhart et al., 1998). In response to the general concern generated by the widespread prevalence of frog populations with a high incidence of malformations, Burkhart et al. (1998) conducted an extensive evaluation of water quality using FETAX. Water and sediment samples were collected from ponds in Minnesota with high incidences of frog malformations and from ponds with unaffected frog populations. Pond water from affected sites produced a high frequency of malformations and mortality in *X. laevis*. Removal of microbial contamination by boiling and filtration had no affect on the results. The teratogenic/toxic activities of the water samples were reduced or eliminated when samples were passed through activated carbon (Burkhart et al., 1998). The results of the studies excluded ion concentration, the presence of metals, and infectious organisms as causal factors of abnormal development, and suggested that the water contained one or more unknown agents that induce developmental abnormalities.

Fort et al. (1999b) used FETAX to evaluate the causal factors associated with developmental anomalies in *X. laevis* exposed to these pond water and aqueous sediment extracts. The craniofacial defects and abnormal eye and mouth development were reduced when some pond water and sediment extract samples underwent microfiltration and/or C_{18}-SPE treatments. Ion exchange was also effective in reducing the malformation-inducing activity of some samples. Results suggested that a mixture of naturally occurring compounds (e.g., pesticides) and anthropogenic organic compounds were primarily responsible for the abnormalities observed (Fort et al., 1999b).

16.4.2 FETAX Testing with Soil Samples

FETAX was used to assess the comparative environmental hazard of soil samples from two waste located in the state of Washington, U.S. (Fort et al., 1996). One waste site was contaminated with polycyclic aromatic hydrocarbons (PAHs), while the other was contaminated with heavy metals. An integrated hazard assessment study was conducted with the aqueous extracts of these samples using FETAX, the conventional *Pimephales promelas* 7-day teratogenicity test, and an abbreviated *P. promelas* teratogenicity test using the general FETAX protocol. Because inadequate sample volumes were available to perform definitive testing sufficient to define the EC_{50}, LC_{50}, or MCIG, the decision criteria used was based on rates of mortality, malformations, and growth inhibition. Zinc, copper, and pentachlorophenol were used as reference toxicants. Results from the studies with the aqueous soil extracts indicated that each of the two sample sites induced a contaminant-related increase in the rates of malformations and mortality in both species. Extracts from the site contaminated with PAHs tended to induce greater levels of embryo mortality in both species, with *P. promelas* being somewhat more sensitive. The types of malformations induced by the aqueous extracts from the PAH and heavy metal contaminated sites in *X. laevis* were characteristic of those induced by pentachlorophenol and zinc, respectively. Concurrent with these studies, a battery of bioassays, including lettuce seed (germination), earthworm (survival), *Daphnia* (survival) and larval *Pimephales* (survival) were also performed. In comparison with the other bioassays, the investigators concluded that FETAX and the *P. promelas* developmental toxicity test appeared to be the most predictive of the contaminated samples. Based on the results obtained, Fort et al. (1996) concluded that FETAX is useful as a component of a multi-testing approach to ecotoxicological hazard assessment.

In an extension of the Fort et al. (1996) study describe above, FETAX was used to evaluate the developmental toxicity of aqueous extracts of soil samples collected at six selected waste sites in the state of Washington (Fort et al., 1995). The waste sites included two sites contaminated with metals (copper, lead, zinc; and arsenic, lead, and mercury, respectively), one site contaminated with PAHs, two sites contaminated by petroleum products, and one site contaminated with organochlorine pesticides. Three to five samples from each site, representing baseline and increasing levels of contamination, were collected. Aqueous extracts of the soil samples were prepared and tested in FETAX.

FETAX was conducted in general compliance with the ASTM FETAX Guideline (ASTM, 1991). The concurrent controls consisted of FETAX Solution, whole blasting sand, whole reference soil, extracted blasting sand, and extracted reference soil. Because inadequate sample volumes were available to perform definitive testing sufficient to define the EC_{50}, LC_{50}, or MCIG, the decision criteria used was based on rates of mortality, malformations, and growth inhibition. Samples collected from the PAH- and petroleum product-contaminated sites were more toxic, although malformations were observed also. The metal-contaminated sites induced more malformations, but less toxicity, than the other sites. The organochlorine pesticide-contaminated site samples caused significant levels of embryonic deformities but not mortality.

Consistent with the other study (Fort et al., 1996), FETAX was concluded by the investigators to be more sensitive than other bioassays (lettuce seed, earthworm, *Daphnia*, larval *Pimephales*) in detecting ecotoxicological hazard. Fort et al. (1995) also concluded that FETAX was sensitive enough to detect low levels of developmental abnormalities but robust enough to be suitable for the testing of aqueous soil extracts.

In an effort to determine the significance of experimental design on the results of laboratory soil toxicity studies with FETAX, two different sample preparations were evaluated from three contaminated waste sites (Fort and Stover, 1997). Whole soil and aqueous soil extracts from each site were evaluated. Site 1 soil was characterized as loamy with a relatively high total organic carbon (TOC), moisture fraction (MF), and sulfide content. This site was contaminated with organochlorine pesticides. Site 2 soil, contaminated with PAHs and pentachlorophenol, was characterized as silt/clay with low/moderate TOC, MF, and sulfides. The Site 3 soil sample consisted of two separate sub-site samples. The first sub-site sample "a" was characterized as loamy with a relatively high TOC, moisture fraction (MF), and sulfide content. The second sub-site sample "b" was characterized as a mixture of silt/clay and sand with relatively low TOC, MF, and sulfide content. Both sub-site samples were contaminated with heavy metals, including copper, lead, and zinc.

The FETAX studies followed the ASTM FETAX Guideline (ASTM, 1991). The concurrent controls consisted of FETAX Solution, whole blasting sand, whole reference soil, extracted blasting sand, and extracted reference soil. A FETAX response was considered indicative of developmental hazard if the TI value was greater than 1.5 or if the $MCIG/LC_{50}$ ratio was less than 0.30. Types of malformations induced were also considered. FETAX testing of the Site 1 sample indicated that substantially greater levels of developmental toxicity were induced by the aqueous extraction of the sample than by the whole bulk soil. Tests with Site 2 samples suggested that both the aqueous extract and the whole bulk sample were capable of inducing comparable rates of developmental toxicity. Tests with sub-site sample “a” of Site 3 indicated that the aqueous extract of the sample induced greater levels of developmental toxicity than the whole soil sample. Toxicity tests with sub-site sample “b” produced variable results that seemed to suggest that the aqueous extract induced greater toxicity than the whole bulk preparations. Fort and Stover (1997) concluded that the results from these studies suggested the importance of experimental design in evaluating potential ecological hazards of contaminated soils, particularly to in regard to amphibian species.

16.4.3 FETAX Testing with Other Environmental Samples

The method was considered useful by Dumont et al. (1983) for determining the teratogenic/embryotoxic potency for a group of chemical mixtures with similar composition that may be expected to be found as environmental pollutants. Tests with five fossil fuel mixtures were conducted using FETAX. The mixtures included a coal-derived fuel oil (Comparative Research Material [CRM]-1), a shale-derived crude (CRM-2), a coal gasifier electrostatic precipitator tar (CRM-4), an aromatic natural petroleum crude (CRM-3), and an aliphatic natural petroleum crude (CRM-5). The experiments were conducted using dilutions of stock solutions made of aqueous extracts of the material. Based on the EC_{50} values, the coal-derived materials were the most teratogenic followed by shale-derived, aromatic, and aliphatic petroleums (**Table 37)**. With respect to embryolethality, CRM-1 and CRM-4 were the most toxic with LC_{50} values of 1.48% and 0.83% respectively followed by CRM-2 with a LC_{50} of 6.97%. CRM-3 and CRM-5 were essentially non-toxic.

These studies demonstrate the utility of FETAX in ecotoxicological hazard assessment, as a means for detecting and prioritizing sites with increased developmental risks. To increase the validity of the interpretation of such data, it would be useful to further evaluate the influence of the physico-chemical properties of environmental samples on the frequency of malformations in

Table 38. Summary of Results for Complex Fossil Fuel Mixtures using FETAX (Dumont et al., 1983).

Comparative Research Material (CRM)	96 Hour Results		TI	Observed Effects
	LC_{50} (%)	EC_{50} (%)		
CRM-1	1.48	0.96	1.54	Growth = 81%; developmental stage attained = 44; pigmentation and motility reduction
CRM-2	6.97	3.36	2.07	Growth = 96%; developmental stage attained = 45/46; pigmentation and motility reduction
CRM-3	33.38	31.10	1.07	Growth = 90%; developmental stage attained = 46
CRM-4	0.83	0.48	1.73	Growth = 87%; developmental stage attained = 45/47; pigmentation and motility reduction
CRM-5 (90% aqueous extract)	--	--	<1.00	Growth = 100%; developmental stage attained = 46/47

FETAX. Additionally, further research is needed on the utility of the current FETAX protocol as an effective assay for assessing water and sediment quality and detecting changes that can have adverse effects on the ecosystem.

16.5 Use of Coded Environmental Samples and Compliance with GLP Guidelines

Generally, coded water/soil/samples were used for ease of identification and chain of custody. These studies were not conducted in compliance with national or international GLP guidelines, nor were they generally conducted at facilities at which GLP studies are normally conducted.

16.6 Availability of Non-Audited FETAX Data

Original data was not sought by NICEATM for any FETAX study involving environmental samples.

16.7 Section 16 Conclusions

Based on the studies evaluated, FETAX appears to be useful in ecotoxicological hazard assessment, and as a means for detecting and prioritizing sites with increased developmental risks. Studies including other bioassays as part of a battery with FETAX (i.e., lettuce seed, earthworm, *Daphnia*, larval *Pimephales*) indicated that FETAX was sensitive enough to detect low levels of developmental abnormalities, but robust enough to be suitable for testing aqueous soil extracts. A comprehensive ASTM protocol for use in such assessments is available (ASTM, 1998). To increase the validity of the interpretation of such data, it would be useful to further evaluate the influence of the physico-chemical properties of environmental samples on the frequency of malformations in FETAX. Additionally, further research on the performance of the current FETAX protocol as an effective assay for assessing water and sediment quality and detecting changes that can have adverse effects on the ecosystem may provide further insight that could optimize ecotoxicological assessments. It may also be helpful to further evaluate how FETAX could best fit into a test battery for prioritizing of sites for further testing and remediation.