

PFOS:
A FROG EMBRYO TERATOGENESIS ASSAY – *XENOPUS*
(FETAX)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-116

3M LAB REQUEST NO. U2723

ASTM Method E 1439-91

AUTHORS:

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STUDY INITIATION DATE: May 11, 2000

STUDY COMPLETION DATE: April 6, 2001

SUBMITTED TO:

3M Corporation
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St. Paul, Minnesota 55144

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-116

STUDY COMPLETION: April 6, 2001

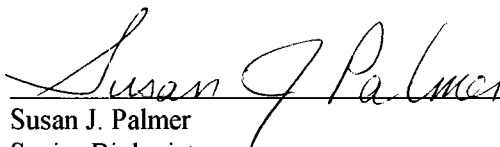
This study was not conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989. Data collection, record keeping, training and facility records at Wildlife International, Ltd. complied with Good Laboratory Practices, with the following exceptions:

The test substance was not characterized in accordance with full GLP compliance prior to the study; however, the characterization was performed according to 3M Standard Operating Procedures and Methods, and all raw data are being maintained in the 3M archives. The test substance has been recharacterized in accordance with GLP on September 7, 2000.

The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

The phase of the study conducted by University of Maryland was not conducted in compliance with Good Laboratory Practices.

STUDY DIRECTOR:



Susan J. Palmer
Senior Biologist

4-6-01

DATE

SPONSOR APPROVAL:



Sponsor

5/15/01

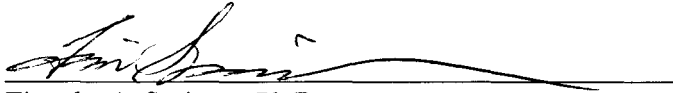
DATE

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	May 12, 2000	May 12, 2000	May 18, 2000
Analytical Data and Draft Report	July 12 & 13, 2000	July 13, 2000	July 14, 2000


Timothy A. Springer, Ph.D.
Manager, Regulatory and Technical Support

4/6/01
DATE

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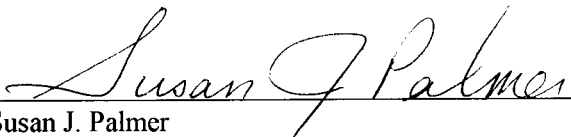
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-116

STUDY DIRECTOR:



Susan J. Palmer
Senior Biologist

4-6-01

DATE

MANAGEMENT:



Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and
Non-Target Plants

4/6/01

DATE

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Rochelle R. Robideau
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	454A-116
STUDY:	PFOS: A Frog Embryo Teratogenesis Assay – <i>Xenopus</i> (FETAX)
TEST SUBSTANCE:	PFOS (Perfluorooctanesulfonate, Potassium Salt) IUPAC Name: 1-Octanesulfonic acid, 1,1,2,2,3,3,4,4,5,5,6,6, 7,7,8,8,8-heptafluoro-potassium salt; CAS #2795-39-3
REFERENCE SUBSTANCE:	6-aminonicotinamide
NOMINAL TEST CONCENTRATIONS:	Negative Control, 1.82, 3.07, 5.19, 8.64, 14.4 and 24.0 mg a.i./L and 5.5 and 2500 mg/L 6-aminonicotinamide
MEAN MEASURED TEST CONCENTRATIONS:	1 st Assay: Negative Control, 2.00, 2.83, 4.73, 7.90, 14.7 and 24.6 mg a.i./L 2 nd Assay: Negative Control, 1.91, 3.04, 4.82, 7.97, 13.3 and 23.1 mg a.i./L 3 rd Assay: Negative Control, 1.93, 3.27, 5.25, 8.26, 14.0 and 23.9 mg a.i./L
TEST DATES:	1 st Assay: Experimental Start – May 15, 2000 Biological Termination – May 19, 2000 Experimental Termination – May 22, 2000 2 nd and 3 rd Assays: Experimental Start – May 22, 2000 Biological Termination – May 26, 2000 Experimental Termination – May 30, 2000
LENGTH OF EXPOSURE:	96 Hours

TEST ORGANISM:	South African Clawed Frog (<i>Xenopus laevis</i>)
SOURCE OF TEST ORGANISMS:	University of Maryland Wye Research and Education Center Queenstown, Maryland 21658
AGE OF TEST ORGANISMS:	Embryos

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SUMMARY

- Continued -

RESULTS (BASED ON MEAN MEASURED CONCENTRATIONS):	<u>1st Assay:</u>	<u>2nd Assay:</u>	<u>3rd Assay:</u>
96-HOUR LC ₅₀ :	13.8 mg a.i./L	17.6 mg a.i./L	15.3 mg a.i./L
95% CONFIDENCE LIMITS:	12.38 – 15.31 mg a.i./L	15.53 – 19.96 mg a.i./L	13.13 – 17.75 mg a.i./L
96-HOUR EC ₅₀ :	12.1 mg a.i./L	17.6 mg a.i./L	16.8 mg a.i./L
95% CONFIDENCE LIMITS:	10.00 – 14.58 mg a.i./L	13.49 – 22.88 mg a.i./L	12.35 – 22.82 mg a.i./L
MINIMUM CONCENTRATION TO INHIBIT GROWTH (MCIG):	Not calculable	7.97 mg a.i./L	8.26 mg a.i./L
TERATOGENIC INDEX (TI):	1.1	1.0	0.9

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INTRODUCTION

This study was conducted in three assays by Wildlife International, Ltd. for 3M Corporation at the University of Maryland Wye Research and Education Center (UM-WREC) in Queenstown, Maryland and at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the first assay was conducted from May 15 to May 19, 2000, while the second and third assays were concurrently conducted from May 22 to May 26, 2000. Raw data generated by Wildlife International, Ltd., data obtained from the University of Maryland, and a copy of the final report are filed under Project Number 454A-116 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the possible developmental toxicity of PFOS (Perfluorooctanesulfonate, Potassium Salt), also referred to as test substance U2723, on the embryos of the South African clawed frog (*Xenopus laevis*) during a 96-hour exposure period.

EXPERIMENTAL DESIGN

Three definitive assays were conducted with PFOS using the frog embryo teratogenesis assay – *Xenopus* (FETAX). During each assay, *Xenopus* embryos were exposed to a geometric series of six test concentrations (PFOS in FETAX solution), a negative control (FETAX solution only), and two concentrations of a reference toxicant (6-aminonicotinamide in FETAX solution) for 96 hours. Two replicate test chambers were maintained in each treatment group and four replicate test chambers were maintained in each control group. Each test chamber contained 25 embryos for a total of 50 embryos per treatment group and 100 embryos per control group. An abiotic replicate at the highest concentration was included in each assay for analytical sampling at 96 hours.

Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 1.82, 3.07, 5.19, 8.64, 14.4 and 24.0 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined at Wildlife International, Ltd. from samples of test solution collected from each treatment and the negative control group prior to and at the end of each assay.

The test solutions were prepared at Wildlife International, Ltd. and were delivered to UM-WREC prior to each assay. At UM-WREC, the embryos were randomly assigned to test chambers containing the appropriate FETAX solution and were observed for 96 hours. The FETAX solutions in the test chambers were renewed every 24 hours, and mortality observations and general water quality measurements were made at each 24-hour renewal period. At the end of the 96-hour exposure period, each surviving embryo was examined for malformations using a dissecting microscope. An LC50 value for mortality and an EC50 value for malformation were calculated at 96 hours for each assay. When possible, the minimum concentration to inhibit growth (MCIG) and the teratogenic index (TI) were calculated for each assay.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)". The protocol was based on procedures outlined in ASTM Standard E1439-91 *Standard Guide for Conducting the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)* (1) and ASTM Standard E729-88a *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (2).

Test and Reference Substances

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International, Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 98.9%, and an expiration date of 2008. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated September 7, 2000 indicated a purity of 86.9% and an expiration date of August 31, 2001. The test substance was stored at ambient room temperature.

The reference substance was maintained by UM-WREC. It was identified as 6-aminonicotinamide (CAS #329-89-5; Sigma; Lot No. 125H0006).

Preparation of Test Concentrations

Prior to each assay, FETAX solution was prepared at UM-WREC and delivered to Wildlife International, Ltd. for the preparation of the stock and test solutions. The second and third assays were conducted concurrently using common test solutions. A primary stock solution was prepared in FETAX solution at a concentration of 48 mg a.i./L. The primary stock solution was mixed by sonication and stirring to aid in the solubilization of the

test substance. The primary stock was proportionally diluted with FETAX solution to prepare the six test concentrations. Nominal test concentrations were 1.82, 3.07, 5.19, 8.64, 14.4 and 24.0 mg a.i./L, based on a test substance purity of 86.9%. The test solutions were delivered to UM-WREC prior to the start of each assay. The reference toxicant solutions were prepared at UM-WREC.

Analytical Chemistry

Samples were collected from each test solution and the negative control solution at preparation, and at the termination of each assay (96 hours) to measure concentrations of the test substance. Samples of test solution from the abiotic replicates also were collected at 96 hours. The samples were analyzed at Wildlife International, Ltd. Analytical procedures used in the analysis of the samples are provided in Appendix II.

In-Life Phase of Assays

Details of the in-life phase of the three assays conducted at UM-WREC are included in Appendix I.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in FETAX solution samples collected during the three assays are presented in Table 1 and in the analytical chemistry report (Appendix II). Nominal concentrations selected for use in this study were 1.82, 3.07, 5.19, 8.64, 14.4 and 24.0 mg a.i./L. In the first assay, the measured concentrations of PFOS in test solution samples collected prior to initiation of exposure of the test organisms ranged from 112 to 141% of the nominal concentrations. Samples collected at test termination had a measured concentration range of 54.7 to 98.6% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present.

For the second and third assays, common test solutions were prepared. The measured concentrations of PFOS in test solution samples collected prior to initiation of exposure of the test organisms ranged from 95.8 to 117% of the nominal concentrations. In the second assay, samples collected at test termination had a measured concentration range of 80.7 to 112% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present. In the third assay, samples collected at test termination had a measured concentration range of 93.4 to 114% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present.

When measured concentrations of the samples analyzed prior to and at the end of each assay were averaged, the mean measured concentrations for the first assay were 2.00, 2.83, 4.73, 7.90, 14.7 and 24.6 mg a.i./L, representing 110, 92.2, 91.1, 91.4, 102 and 103% of nominal concentrations, respectively. The mean measured concentrations for the second assay were 1.91, 3.04, 4.82, 7.97, 13.3 and 23.1 mg a.i./L, representing 105, 99.0, 92.9, 92.2, 92.4 and 96.3% of nominal concentrations, respectively. The mean measured concentrations for the third assay were 1.93, 3.27, 5.25, 8.26, 14.0 and 23.9 mg a.i./L, representing 106, 107, 101, 95.6, 97.2 and 99.6% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations for each assay.

Definitive Assay Results

The results of the three definitive FETAX assays conducted with PFOS at the UM-WREC are included in Appendix I and are summarized in Table 2. Exposure to PFOS caused significant embryo mortality at the two highest test concentrations (14.4 and 24.0 mg a.i./L nominal concentration) in each of the three assays. Most of the embryo mortality appeared to be caused by the gut coiling through the body wall at the two highest concentrations. Embryo survival was $\geq 90\%$ at all test concentrations up to 8.64 mg a.i./L nominal concentration. The 96-hour LC50 values for PFOS ranged from 13.8 to 17.6 mg a.i./L mean measured concentration in the three assays (Table 2).

There was a positive correlation between PFOS exposure and malformations in each of the three assays, with increasing concentrations causing an increase in the incidence of malformed embryos. The 96-hour EC50 values, based on the incidence of malformed embryos, ranged from 12.1 to 17.6 mg a.i./L mean measured concentration in the three assays (Table 2). The most common types of malformations caused by exposure to PFOS were improper gut coiling, edema, notochord abnormalities and facial abnormalities.

Growth of the embryos was not effected by exposure to PFOS in the first assay. In the second and third assays, the minimum concentration inhibiting growth (MCIG) was 8.64 mg a.i./L nominal concentration. This reduction in growth may be attributed more to the increased incidence of malformed embryos in the test concentrations than to an overall decrease in growth. The teratogenic index (TI), defined as the 96-hour LC50 value for mortality divided by the 96-hour EC50 value for malformed embryos, provides an estimate of the teratogenic risk associated with PFOS. The TI's in the three assays ranged from 0.9 to 1.1, indicating the PFOS has a low potential to be a developmental hazard.

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CONCLUSIONS

The 96-hour LC50 value for mortality for *Xenopus laevis* exposed to PFOS in each of three assays was 13.8, 17.6 and 15.3 mg a.i./L mean measured concentration, respectively. The 96-hour EC50 value for embryo malformation in each of three assays was 12.1, 17.6 and 16.8 mg a.i./L mean measured concentration, respectively. The MCIG, determined in the second and third assays, was 7.97 and 8.26 mg a.i./L mean measured concentration, respectively. The TI's ranged from 0.9 to 1.1 in the three assays, indicating the PFOS has a low potential to be a developmental hazard.

REFERENCES

- 1 **ASTM Standard E1439-91.** 1998. *Standard Guide for Conducting the Frog Embryo Teratogenesis Assay – Xenopus (FETAX).* American Society for Testing and Materials. Philadelphia, Pennsylvania.
- 2 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials. Philadelphia, Pennsylvania.

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Table 1
Summary of Analytical Chemistry Data

Nominal Test Concentration (mg a.i./L)	Sampling Time (Day)	1 st Assay			2 nd Assay ¹			3 rd Assay ¹		
		Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	0 ²	<LOQ ⁴	<LOQ	--	<LOQ	<LOQ	--	<LOQ	<LOQ	--
	4 ³	<LOQ			<LOQ			<LOQ		
1.82	0	2.58	2.00	110	1.77	1.91	105	1.77	1.93	106
	4	1.42			2.04			2.08		
3.07	0	3.94	2.83	92.2	3.59	3.04	99.0	3.59	3.27	107
	4	1.72			2.49			2.94		
5.19	0	6.62	4.73	91.1	5.45	4.82	92.9	5.45	5.25	101
	4	2.84			4.18			5.05		
8.64	0	10.7	7.90	91.4	8.43	7.97	92.2	8.43	8.26	95.6
	4	5.09			7.51			8.09		
14.4	0	18.5	14.7	102	14.5	13.3	92.4	14.5	14.0	97.2
	4	10.8			12.1			13.5		
24.0	0	26.9	24.6	103	23.0	23.1	96.3	23.0	23.9	99.6
	4	22.3			23.1			24.7		
24.0 (abiotic)	4	23.7	--	98.6	23.9	--	99.6	24.1	--	100

¹ The 2nd and 3rd assays were conducted concurrently using common batches of test solution prepared for Day 0.

² Day 0 samples were collected from the test solutions at preparation prior to transfer to UM-WREC.

³ Day 4 samples were collected from the test solutions at the termination of each assay at UM-WREC and transferred to Wildlife International, Ltd. for analysis.

⁴ The limit of quantitation (LOQ) was 0.240 mg a.i./L.

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Table 2

LC50, EC50, MCIG and TI Values¹

Parameter	1 st Assay	2 nd Assay	3 rd Assay
96-Hour LC50 (mg a.i./L):	13.8	17.6	15.3
95% Confidence Limits (mg a.i./L):	12.38 – 15.31	15.53 – 19.96	13.13 – 17.75
96-Hour EC50 (mg a.i./L):	12.1	17.6	16.8
95% Confidence Limits (mg a.i./L):	10.00 – 14.58	13.49 – 22.88	12.35 – 22.82
Minimum Concentration Inhibiting Growth (MCIG) (mg a.i./L):	-- ²	7.97	8.26
Teratogenic Index (TI):	1.1	1.0	0.9

¹ Values are based on the mean measured concentrations for each assay.

² The MCIG could not be calculated because PFOS had no effect on embryonic growth at any of the test concentrations with surviving embryos in the 1st assay.

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APPENDIX I

Certificate of Analysis

INTERIM CERTIFICATE OF ANALYSIS

Revision 1(9/7/00)

Centre Analytical Laboratories COA Reference #: 023-018A

3M Product: PFOS, Lot 217

Reference #: SD-018

Purity: 86.9%

Test Name	Specifications	Result
Purity ¹		86.9%
Appearance	White Crystalline Powder	Conforms
Identification NMR		Positive
Metals (ICP/MS)		
1. Calcium		1. 0.005 wt./wt. %
2. Magnesium		2. 0.001 wt./wt. %
3. Sodium		3. 1.439 wt./wt. %
4. Potassium ²		4. 6.849 wt./wt. %
5. Nickel		5. <0.001 wt./wt. %
6. Iron		6. 0.005 wt./wt. %
7. Manganese		7. <0.001 wt./wt. %
Total % Impurity (NMR)		1.93 wt./wt. %
Total % Impurity (LC/MS)		8.41 wt./wt. %
Total % Impurity (GC/MS)		None Detected
Related Compounds – POAA		0.33 wt./wt. %
Residual Solvents (TGA)		None Detected
Purity by DSC		Not Applicable ³
Inorganic Anions (IC)		
1. Chloride		1. <0.015 wt./wt. %
2. Fluoride		2. 0.59 wt./wt. %
3. Bromide		3. <0.040 wt./wt. %
4. Nitrate		4. <0.009 wt./wt. %
5. Nitrite		5. <0.006 wt./wt. %
6. Phosphate		6. <0.007 wt./wt. %
7. Sulfate ⁴		7. 8.76 wt./wt. %
Organic Acids ⁵ (IC)		
1. TFA		1. <0.1 wt./wt. %
2. PFPA		2. <0.1 wt./wt. %
3. HFBA		3. 0.10 wt./wt. %
4. NFPA		4. 0.28 wt./wt. %
Elemental Analysis ⁶ :		
1. Carbon	1. Theoretical Value = 17.8%	1. 12.48 wt./wt. %
2. Hydrogen	2. Theoretical Value = 0%	2. 0.244 wt./wt. %
3. Nitrogen	3. Theoretical Value = 0%	3. 1.74 wt./wt. %
4. Sulfur	4. Theoretical Value = 5.95%	4. 8.84 wt./wt. %
5. Fluorine	5. Theoretical Value = 60%	5. 54.1 wt./wt. %

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INTERIM CERTIFICATE OF ANALYSIS
Centre Analytical Laboratories COA Reference #: 023-018A

Date of Last Analysis: 08/31/00

Expiration Date: 08/31/01

Storage Conditions: Frozen $\leq -10^{\circ}\text{C}$

Re-assessment Date: 08/31/01

¹Purity = 100% - (sum of metal impurities, 1.45% + LC/MS impurities, 8.41% + Inorganic Fluoride, 0.59% + NMR impurities, 1.93% + organic acid impurities, 0.38% + POAA, 0.33%)

Total impurity from all tests = 13.09%

Purity = 100% - 13.09% = 86.9%

²Potassium is expected in this salt form and is therefore not considered an impurity.

³Purity by DSC is generally not applicable to materials of low purity. No endotherm was observed for this sample.

⁴Sulfur in the sample appears to be converted to SO_4 and hence detected using the inorganic anion method conditions. The anion result agrees well with the sulfur determination in the elemental analysis, lending confidence to this interpretation. Based on the results, the SO_4 is not considered an impurity.

⁵ TFA	Trifluoroacetic acid
HFBA	Heptafluorobutyric acid
NFPA	Nonofluoropentanoic acid
PFPA	Pentafluoropropanoic acid

⁶Theoretical value calculations based on the empirical formula, $\text{C}_8\text{F}_{17}\text{SO}_3\text{K}^+$ (MW=538)

This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 160).

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INTERIM CERTIFICATE OF ANALYSIS
Centre Analytical Laboratories COA Reference #: 023-018A

LC/MS Purity Profile:

Impurity	wt./wt. %
C4	1.22
C5	1.33
C6	4.72
C7	1.14
Total	8.41

Note: The C4 and C6 values were calculated using the C4 and C6 standard calibration curves, respectively. The C5 value was calculated using the average response factors from the C4 and C6 standard curves. Likewise, the C7 value was calculated using the average response factors from the C6 and C8 standard curves.

Prepared By: _____
David S. Bell
Scientist, Centre Analytical Laboratories

Date

Reviewed By: _____
John Flaherty
Laboratory Manager, Centre Analytical Laboratories

Date

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APPENDIX II

Evaluation of the Toxicity and Teratogenicity of Chemical U2723
Using the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)

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Report No.
WREC-00-02

**EVALUATION OF THE TOXICITY AND TERATOGENICITY OF
CHEMICAL U2723 USING THE FROG EMBRYO
TERATOGENESIS ASSAY-XENOPUS (FETAX)**

Final Report

Prepared for

Wildlife International, Ltd.
8598 Commerce Drive
Easton, MD 21601

Prepared by

Steven D. Turley
and
Dennis T. Burton

University of Maryland
Wye Research and Education Center
P.O. Box 169
Queenstown, MD 21658

July 15, 2000

Revision Date:

March 19, 2001

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1. INTRODUCTION

The evaluation of the potential toxicity and teratogenicity of chemical U2723 was initiated at the request of Mr. David A. Palmer, Wildlife International, Ltd., Easton, Maryland. The study was conducted by Mr. Steven D. Turley and Dr. Dennis T. Burton of the University of Maryland Wye Research and Education Center, Queenstown, Maryland. The test program was performed in accordance with the "Revised Statement of Work for Evaluating the Potential Teratogenicity of U2723 Using 96-Hour Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)", submitted to and approved by Wildlife International, Ltd., March 30, 2000 (Turley, 2000). The potential toxicity and teratogenicity of chemical U2723 was evaluated by the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Three 96-h definitive FETAX assays were conducted on chemical U2723.

2. MATERIALS AND METHODS

Three definitive FETAX assays were conducted on chemical U2723 using the frog embryo teratogenesis assay-*Xenopus* (FETAX). The assay is a 96-h quantitative developmental assay used to screen for developmental toxicants in aquatic media. The assays were conducted using the static renewal (solutions renewed every 24 h) test protocol Designation E 1439-91 of the American Society for Testing and Materials (ASTM, 1998). Embryo lethality (96-h LC50), malformations (96-h EC50), and growth retardation were used as endpoints in the assays.

Embryos were obtained from breeding colonies of *Xenopus laevis* at the University of Maryland Wye Research and Education Center (UMD/WREC). The UMD/WREC adult colonies were maintained in flow-through (~4 replacement volumes per day) circular polyethylene aquaria (0.91 m I.D. x 0.36 m high) with a water depth of 10 cm. Each aquarium contained a maximum of 10 adults. UMD/WREC non-chlorinated deep well water held at 23.5 ± 0.5 °C served as the culture medium. All frogs were fed every 5-6 d with commercial beef liver supplemented with liquid vitamins (PolyViSol™; Mead-Johnson Nutritionals, Evansville, Indiana). The colony was held under a photoperiod of 16 h light:8 h dark. Mating pairs were bred in the dark in 23.5 ± 0.5 °C UMD/WREC non-chlorinated water at ~70 d intervals by injecting 400 and 800 I.U. of human chorionic gonadotropin (HCG) in the dorsal lymph sac of the males and females, respectively. Amplexus occurred 4-6 h after injecting HCG; egg deposition occurred 9-12 h following HCG injection.

Embryos between normal stage 8 blastulae and normal stage 11 gastrulae were used to initiate the assays (Nieuwkoop and Faber, 1975). Embryos from separate breeding pairs were used for each test to check for variability in the sensitivity of individual clutches of embryos. All embryos were examined two times for viability. The embryos were de-jellied in a 2% L-cysteine solution (2 g of L-cysteine per 98 mL of FETAX solution). Once de-jellied, the embryos were rinsed and re-suspended in FETAX solution (ASTM, 1998). The embryos were assayed in covered 60 mm glass Petri dishes containing 10 mL of solution. All embryos were randomly assigned to the Petri dishes. Two replicates of 25 embryos/replicate were used for each of six assay treatments; four replicates were used for the controls as required by the test protocol (ASTM, 1991). The assays were conducted at 24 ± 0.2 °C under a 12-h light: 12-h dark photoperiod (fluorescent lights; ~75 foot candles at the surface of the test medium) in a constant temperature environmental chamber. All Petri dishes were randomly placed in the environmental chamber used for the assays. Wildlife International, Ltd., personnel prepared and delivered stock solutions of each concentration at the start of each assay. The concentration of each solution was quantified at Wildlife International, Ltd. Two concentrations of the reference toxicant, 6-aminonicotinamide (CAS #329-89-5; Sigma; Lot No. 125H0006), were used at each assay period as outlined in the ASTM (1998) protocol.

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Mortality observations and general water quality measurements were made at each 24-h renewal period. All dead embryos were removed at each observation period. At the end of the 96-h exposure period, each surviving embryo was examined for malformations using a dissecting microscope. The identification and interpretation of malformations in the embryos at 96 h were made via the atlas of Bantle et al. (1991). The head-tail length of each surviving embryo was determined via Sigma Scan's digitizing software (SPSS, Corte Madera, California). A random group of normal embryos and embryos with each type of malformation were preserved in 10% buffered formalin (pH 7) for photographing.

The Trimmed Spearman-Kärber statistical procedure was used to determine the 96-h LC50 for mortality and 96-h EC50 for malformations. The minimum concentration to inhibit growth (MCIG) was determined by Bonferroni's T-Test. All statistical tests were performed using Toxstat (WEST and Gulley, 1994). A minimum probability level of 0.05 was used. The teratogenic index (TI) was calculated by dividing the LC50 by the EC50. The test acceptability criteria given in the ASTM (1998) test protocol was used for each definitive test.

3. RESULTS AND DISCUSSION

The data for definitive assays 1, 2, and 3 are summarized in Appendices 1, 2, and 3, respectively. Copies of the raw data sheets and 6-aminonicotinamide reference toxicant data for assays 1, 2, and 3 are given in Appendices 4, 5, and 6, respectively. The nominal concentrations of 1.9, 3.2, 5.4, 9.0, 15.0 and 25.0 mg/L on the raw data sheets correspond to the nominal concentrations of 1.82, 3.07, 5.19, 8.64, 14.4 and 24.0 mg a.i./L in the report. Photographs of normal and malformed embryos of each type observed in the study are given in Attachment 1. Table 1 is summary of the endpoint parameters established in the three definitive assays.

Table 1. Summary of Data for the Three Assays with Chemical U2723^a

Parameter	Assay 1	Assay 2	Assay 3
LC50 (95% C. I.)	13.8 (12.38 - 15.31)	17.6 (15.53 - 19.96)	15.3 (13.13 - 17.75)
EC50 (95% C. I.)	12.1 (10.00 - 14.58)	17.6 (13.49 - 22.88)	16.8 (12.35 - 22.82)
MCIG	^b	7.97	8.26
TI	1.1	1.0	0.9

^a The LC50, EC50, and MCIG values are based on mean measured concentrations of chemical U2723 (mg a.i./L) for each assay.

^b The MCIG could not be calculated because chemical U2723 had no effect on embryonic growth at any of the test treatments with surviving embryos (Appendix A; Table A1- 4).

Exposure to chemical U2723 caused significant embryo mortality at the two highest treatment concentrations (14.4 and 24.0 mg/L) in each of the three assays. Most of the embryo mortality appeared to be caused by the gut coiling through the body wall at the two highest assay treatments. Embryo survival was ≥90% at all exposure treatments up to 8.64 mg/L. The 96-h LC50s for chemical U2723 ranged from 13.8 to 17.6 mg/L (Table 1).

There was a positive correlation between chemical U2723 exposure and malformations in each of the three assays, with increasing concentrations causing an increase in the incidence of malformed embryos. The 96-h EC50s, based on the incidence of malformed embryos, ranged from 12.1 to 17.6 mg/L (Table 1). The most common types of malformations caused by exposure to chemical U2723 were: improper gut coiling,

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edema, notochord abnormalities and facial abnormalities.

Growth of the embryos was not affected by exposure to chemical U2723 in assay 1. In assays 2 and 3, the minimum concentration inhibiting growth (MCIG) was 7.97 and 8.26 mg/L, respectively. This reduction in growth may be attributed more to the increased incidence of malformed embryos in these test concentrations than an overall decrease in growth. Malformed embryos are generally smaller than normally developing embryos.

The teratogenic index (TI), which by definition is the 96-h LC50 divided by the 96-h EC50 (malformed embryos), provides an estimate of the teratogenic risk associated with a material (Dumont et al., 1983). TI values of 1.5 to 2.0 indicate that a material may be a potential teratogen. Materials with TI values >2.0 should be considered for further teratogenicity testing. The TI's in the current study ranged from 0.9 to 1.1 which indicate that chemical U2723 has a low potential to be a developmental hazard.

The three assays met all of the ASTM (1998) protocol test acceptability criteria, with one exception. The reference toxicant (6-aminonicotinamide) high concentration of 2,500 mg/L did not meet the ASTM protocol test acceptability criterion. A concentration of 2,500 mg/L should kill between 40 and 60% of the embryos in 96 h. All embryos died at 2,500 mg/L in all three reference toxicant tests. All three reference toxicant tests met the protocol test acceptability criteria for the low reference toxicant concentration of 5.5 mg/L which should cause between 40 and 60% malformed embryos after 96 h of exposure. The 5.5 mg/L reference toxicant tests produced an average of 55.6, 55.3, and 53.1% malformed embryos during assays 1, 2, and 3, respectively. The results of the reference toxicant concentrations in the three current tests are consistent and not at variance with previous experience in our laboratory. The survival of control embryos after 96 hours in our laboratory consistently meet the ASTM protocol acceptability criterion of >90% survival. In the current chemical U2723 assays, the average survival of the controls was 99, 99, and 100%, in assays 1, 2, and 3, respectively.

The chain of custody forms for delivery of the assay solutions from Wildlife International, Ltd. to the UMD/WREC were retained by Wildlife International, Ltd. Thus, copies of the chain of custody forms are not included in this report.

4. REFERENCES

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APPENDIX 1

**FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX) CONDUCTED
WITH CHEMICAL U2723 (ASSAY 1)**

Test Method:	ASTM Designation E 1439-91 ASTM (1998)
Type of Test:	Static renewal (every 24 h)
Date:	May 15-19, 2000
Investigator:	S. D. Turley
Laboratory:	UMD/WREC
Chemical:	
Designation:	U2723
Source:	Wildlife International, Ltd.
General Water Quality:	See Table A1-1
Test Medium:	FETAX solution
Test Organism:	
Scientific Name:	<i>Xenopus laevis</i>
Age at Start of Test:	Stage 8 blastula to stage 11 gastrula
Source:	UMD/WREC culture
Experimental Chambers:	
Material:	Glass petri dishes
Test Solution Volume:	10 mL
No. Organisms/Replicate:	25
No. Organisms/Treatment:	Control: 100 Treatment: 50
Lighting:	Fluorescent; 60-85 foot candles
Endpoints:	Mortality; malformation; growth
Test Temperature:	24 ± 2°C

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Results:

Note: For Assay 1, statistical calculations are based on the mean measured concentrations of 2.00, 2.83, 4.73, 7.90, 14.70 and 24.60 mg a.i./L, rather than on the nominal concentrations.

Mortality:

The 96-h LC50 = 13.8 mg a.i./L (95% confidence limits = 12.38 - 15.31).

The mortality data are summarized in Table A1-2.

Malformations:

The 96-h EC50 (malformed embryos) = 12.1 mg a.i./L (95% confidence limits = 10.00 - 14.58).

The number of malformed embryos are summarized in Table A1-2. The types of malformations are summarized in Table A1-3.

Growth:

Chemical U2723 had no effect on embryonic growth at any of the test treatments with surviving embryos. As a result, the minimum concentration inhibiting growth (MCIG) could not be calculated.

The growth data are summarized in Table A1-4.

Teratogenic Index:

The teratogenic index (TI) = 1.1.

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Table A1-1. Summary of The General Water Chemistry Data for Chemical U2723 - Assay 1 - Dissolved Oxygen (mg/L)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	8.3	8.4	8.4	8.4	8.4	8.4	8.5
<u>Day 1</u>							
24 H	8.0	7.8	7.9	7.9	8.0	8.0	7.9
0 H	8.4	8.4	8.5	8.4	8.5	8.5	8.4
<u>Day 2</u>							
24 H	7.7	7.7	7.6	7.7	7.8	7.8	7.8
0 H	8.3	8.3	8.4	8.3	8.2	8.3	8.3
<u>Day 3</u>							
24 H	7.4	7.5	7.7	7.7	7.8	7.9	7.9
0 H	8.3	8.2	8.1	8.2	8.1	8.1	8.1
<u>Day 4</u>							
24 H	7.7	7.7	7.6	7.5	7.7	7.7	7.7
Mean	8.1	8.0	8.1	8.1	8.1	8.1	8.1
Min	7.4	7.5	7.6	7.7	7.8	7.8	7.8
Max	8.4	8.4	8.5	8.4	8.5	8.5	8.5

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Table A1-1. (Continued) - pH (Standard Units)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	7.74	7.78	7.79	7.78	7.78	7.76	7.62
<u>Day 1</u>							
24 H	7.26	7.24	7.30	7.34	7.35	7.34	7.33
0 H	7.24	7.45	7.38	7.43	7.43	7.44	7.32
<u>Day 2</u>							
24 H	7.16	7.17	7.20	7.23	7.23	7.23	7.25
0 H	7.22	7.25	7.28	7.32	7.34	7.35	7.27
<u>Day 3</u>							
24 H	7.11	7.13	7.16	7.17	7.25	7.18	6.95
0 H	7.13	7.20	7.23	7.25	7.26	7.26	7.18
<u>Day 4</u>							
24 H	7.11	7.15	7.18	7.19	7.18	7.20	7.19
Min	7.11	7.13	7.16	7.17	7.18	7.18	6.95
Max	7.74	7.78	7.79	7.78	7.78	7.76	7.62

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Table A1-1. (Continued) - Temperature (°C)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 1</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 2</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 3</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 4</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Mean	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Min	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Max	24.0	24.0	24.0	24.0	24.0	24.0	24.0

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Table A1-2. FETAX Toxicity Test Data - Percent Embryo Mortality and Malformations after 96 Hours of Exposure to Chemical U2723 - Assay 1

Concentration (mg/L)	Rep	Number Embryos Alive	Percent Mortality	Number Embryos Malformed	Percent Malformed
Control	1	24	4.0	1	4.2
	2	25	0.0	1	4.0
	3	25	0.0	1	4.0
	4	25	0.0	1	4.0
1.82	1	24	4.0	2	8.3
	2	25	0.0	2	8.0
3.07	1	24	4.0	2	8.3
	2	24	4.0	5	20.8
5.19	1	23	8.0	7	30.4
	2	22	12.0	3	13.6
8.64	1	21	16.0	4	19.0
	2	23	8.0	7	30.4
14.40	1	16	36.0	9	56.3
	2	15	40.0	11	73.3
24.00	1	0	100		
	2	0	100		

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Table A1-3. FETAX Toxicity Test Data - Type and Number of Malformed Embryos after 96 Hours Exposure to Chemical U2723 - Assay 1

Malformation	Test Concentration (mg/L)															
	0				1.82				3.07				5.19			
	Rep				Rep				Rep				Rep			
	1	2	3	4	1	2	1	2	1	2	1	2	1	2	1	2
Severe													1			
Stunted																
Gut	1	1	1	1	1	1	2	5	7	3	4	5	7	11		
Edema:																
Multiple								2	2		2	2	4	3		
Cardiac		1					1	3	1		2	2	4	1		
Abdominal							1	3	1		2	1	3	2		
Facial								2	2		2	2	4	2		
Cephalic																
Optic								2	2		1	1	2	6		
Tail																
Notochord													1	1		
Fin																
Face	1	1		1	2	2	1	1	4	3	2	3	4	6		
Eye													1	1		
Brain																
Hemorrhage																
Cardiac						1			2				3	1		
Blisters																
Other*																
# Malformed	1	1	1	1	2	2	2	5	7	3	4	7	9	11		

* Anal edema.

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Table A1-4. FETAX Toxicity Test Data - Embryonic Growth (Length) after 96 Hours
Exposure to Chemical U2723 - Assay 1

Concentration (mg/L)	Rep	Number Embryos Alive	Average Length (mm)	Mean Average Length (mm)
Control	1	24	9.05	8.59
	2	25	8.40	
	3	25	8.29	
	4	25	8.62	
1.82	1	24	8.44	8.29
	2	25	8.14	
3.07	1	24	8.88	8.80
	2	24	8.72	
5.19	1	23	8.42	8.51
	2	22	8.60	
8.64	1	21	8.83	8.71
	2	23	8.58	
14.40	1	16	8.41	8.08
	2	15	7.74	
24.00	1	0		
	2	0		

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APPENDIX 2

FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX) CONDUCTED
WITH CHEMICAL U2723 (ASSAY 2)

Test Method:	ASTM Designation E 1439-91 ASTM (1998)
Type of Test:	Static renewal (every 24 h)
Date:	May 22-26, 2000
Investigator:	S. D. Turley
Laboratory:	UMD/WREC
Chemical:	
Designation:	U2723
Source:	Wildlife International, Ltd.
General Water Quality:	See Table A2-1
Test Medium:	FETAX solution
Test Organism:	
Scientific Name:	<i>Xenopus laevis</i>
Age at Start of Test:	Stage 8 blastula to stage 11 gastrula
Source:	UMD/WREC culture
Experimental Chambers:	
Material:	Glass petri dishes
Test Solution Volume:	10 mL
No. Organisms/Replicate:	25
No. Organisms/Treatment:	Control: 100 Treatment: 50
Lighting:	Fluorescent; 60-85 foot candles
Endpoints:	Mortality; malformation; growth
Test Temperature:	24 ± 2°C

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Results:

Note: For Assay 2, statistical calculations are based on the mean measured concentrations of 1.91, 3.04, 4.82, 7.97, 13.30 and 23.10 mg a.i./L, rather than on the nominal concentrations.

Mortality:

The 96-h LC50 = 17.6 mg a.i./L (95% confidence limits = 15.53 -19.96).

The mortality data are summarized in Table A2-2.

Malformations:

The 96-h EC50 (malformed embryos) = 17.6 mg a.i./L (95% confidence limits = 13.49 - 22.88)

The number of malformed embryos are summarized in Table A2-2. The types of malformations are summarized in Table A2-3.

Growth:

The minimum concentration inhibiting growth (MCIG) =7.97 mg a.i./L.

The growth data are summarized in Table A2-4.

Teratogenic Index:

The teratogenic index (TI) = 1.0.

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Table A2-1. Summary of The General Water Chemistry Data for Chemical U2723 - Assay 2 - Dissolved Oxygen (mg/L)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	8.2	8.2	8.1	8.2	8.1	8.2	8.2
<u>Day 1</u>							
24 H	8.3	8.3	8.0	8.1	8.1	8.1	8.2
0 H	8.2	8.2	8.2	8.2	8.1	8.1	8.2
<u>Day 2</u>							
24 H	7.6	7.8	7.5	7.2	7.2	7.2	7.1
0 H	8.1	8.1	8.0	8.1	8.0	8.0	8.1
<u>Day 3</u>							
24 H	7.5	7.6	7.5	7.6	7.7	7.3	7.2
0 H	8.1	8.2	8.1	8.1	8.0	8.1	8.0
<u>Day 4</u>							
24 H	7.6	7.6	7.5	7.5	7.6	7.5	7.5
Mean	8.0	8.0	7.9	7.9	7.9	7.8	7.8
Min	7.5	7.6	7.5	7.2	7.2	7.2	7.1
Max	8.3	8.3	8.2	8.2	8.1	8.2	8.2

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Table A2-1. (Continued) - pH (Standard Units)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	7.14	7.24	7.31	7.33	7.37	7.40	7.37
<u>Day 1</u>							
24 H	7.12	7.17	7.21	7.23	7.24	7.26	7.27
0 H	7.11	7.21	7.28	7.35	7.35	7.39	7.34
<u>Day 2</u>							
24 H	7.18	7.22	7.23	7.24	7.24	7.23	7.24
0 H	7.15	7.23	7.30	7.37	7.38	7.41	7.32
<u>Day 3</u>							
24 H	7.20	7.20	7.22	7.25	7.24	7.23	7.25
0 H	7.17	7.21	7.26	7.34	7.36	7.40	7.35
<u>Day 4</u>							
24 H	7.11	7.21	7.20	7.29	7.33	7.39	7.34
Min	7.11	7.17	7.20	7.23	7.24	7.23	7.24
Max	7.20	7.24	7.31	7.37	7.38	7.41	7.37

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Table A2-1. (Continued) - Temperature (°C)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 1</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 2</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 3</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 4</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Mean	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Min	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Max	24.0	24.0	24.0	24.0	24.0	24.0	24.0

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Table A2-2. FETAX Toxicity Test Data - Percent Embryo Mortality and Malformations after 96 Hours of Exposure to Chemical U2723 - Assay 2

Concentration (mg/L)	Rep	Number Embryos Alive	Percent Mortality	Number Embryos Malformed	Percent Malformed
Control	1	25	0.0	1	4.0
	2	25	0.0	2	8.0
	3	25	0.0	0	0.0
	4	24	4.0	1	4.2
1.82	1	25	0.0	2	8.0
	2	25	0.0	2	8.0
3.07	1	23	8.0	2	8.7
	2	22	12.0	0	0.0
5.19	1	24	4.0	1	4.2
	2	22	12.0	4	18.2
8.64	1	22	12.0	4	18.2
	2	23	8.0	5	21.7
14.40	1	22	12.0	4	18.2
	2	13	48.0	9	69.2
24.00	1	9	64.0	6	66.7
	2	6	76.0	4	66.7

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Table A2-3. FETAX Toxicity Test Data - Type and Number of Malformed Embryos after 96 Hours Exposure to Chemical U2723 - Assay 2

Malformation	Test Concentration (mg/L)																
	0				1.82		3.07		5.19		8.64		14.40		24.00		
	Rep				Rep		Rep		Rep		Rep		Rep		Rep		
	1	2	3	4	1	2	1	2	1	2	1	2	1	2	1	2	
Severe																	
Stunted																	
Gut		1			1	1	1				4	1	3	2	6	6	4
Edema:																	
Multiple					2					2		1				3	
Cardiac					2					2	2	1				4	
Abdominal					2					3		1	1			3	
Facial					2				1	3		1				3	
Cephalic																	
Optic					2					1						3	
Tail																	
Notochord				1		1	2							1	1		
Fin																	
Face	1	2				1				2	2	3	1	9	1	3	
Eye																	
Brain																	
Hemorrhage																	
Cardiac		2															
Blisters																	
Other*											1			2			
# Malformed	1	2	0	1	2	2	2	0	1	4	4	5	4	9	6	4	

* Anal edema.

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Table A2-4. FETAX Toxicity Test Data - Embryonic Growth (Length) after 96 Hours
Exposure to Chemical U2723 - Assay 2

Concentration (mg/L)	Rep	Number Embryos Alive	Average Length (mm)	Mean Average Length (mm)
Control	1	25	8.94	8.88
	2	25	8.81	
	3	25	8.90	
	4	24	8.88	
1.82	1	25	8.39	8.45
	2	25	8.50	
3.07	1	23	8.42	8.57
	2	22	8.71	
5.19	1	24	8.69	8.72
	2	22	8.74	
8.64	1	22	7.94	7.93*
	2	23	7.92	
14.40	1	22	7.89	7.51*
	2	13	7.13	
24.00	1	9	7.50	7.39*
	2	6	7.27	

* Significantly different at alpha = 0.05 (Bonferroni T-Test)

APPENDIX 3

**FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX) CONDUCTED
WITH CHEMICAL U2723 (ASSAY 3)**

Test Method:	ASTM Designation E 1439-91 ASTM (1998)
Type of Test:	Static renewal (every 24 h)
Date:	May 22-26, 2000
Investigator:	S. D. Turley
Laboratory:	UMD/WREC
Chemical:	
Designation:	U2723
Source:	Wildlife International, Inc.
General Water Quality:	See Table A3-1
Test Medium:	FETAX solution
Test Organism:	
Scientific Name:	<i>Xenopus laevis</i>
Age at Start of Test:	Stage 8 blastula to stage 11 gastrula
Source:	UMD/WREC culture
Experimental Chambers:	
Material:	Glass petri dishes
Test Solution Volume:	10 mL
No. Organisms/Replicate:	25
No. Organisms/Treatment:	Control: 100 Treatment: 50
Lighting:	Fluorescent; 60-85 foot candles
Endpoints:	Mortality; malformation; growth
Test Temperature:	24 ± 2°C

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Results:

Note: For Assay 3, statistical calculations are based on the mean measured concentrations of 1.93, 3.27, 5.25, 8.26, 14.00 and 23.90 mg a.i./L, rather than on nominal concentrations.

Mortality:

The 96-h LC50 = 15.3 mg a.i./L (95% confidence limits = 13.13 - 17.75).

The mortality data are summarized in Table A3-2.

Malformations:

The 96-h EC50 (malformed embryos) = 16.8 mg a.i./L (95% confidence limits = 12.35 - 22.82).

The number of malformed embryos are summarized in Table A3-2. The types of malformations are summarized in Table A3-3.

Growth:

The minimum concentration inhibiting growth (MCIG) = 8.26 mg a.i./L.

The growth data are summarized in Table A3-4.

Teratogenic Index:

The teratogenic index (TI) = 0.9.

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Table A3-1. Summary of The General Water Chemistry Data for Chemical U2723 - Assay 3 - Dissolved Oxygen (mg/L)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	8.2	8.2	8.1	8.2	8.1	8.2	8.2
<u>Day 1</u>							
24 H	8.3	8.3	8.0	8.2	8.2	8.2	8.3
0 H	8.2	8.2	8.2	8.2	8.1	8.1	8.2
<u>Day 2</u>							
24 H	7.4	7.2	7.1	7.0	7.0	7.0	7.0
0 H	8.1	8.1	8.0	8.1	8.0	8.0	8.1
<u>Day 3</u>							
24 H	7.3	7.1	7.1	7.0	7.0	7.1	7.0
0 H	8.1	8.2	8.1	8.1	8.0	8.1	8.0
<u>Day 4</u>							
24 H	7.4	7.5	7.5	7.5	7.6	7.4	7.4
Mean	7.9	7.9	7.8	7.8	7.8	7.8	7.8
Min	7.3	7.1	7.1	7.0	7.0	7.0	7.0
Max	8.3	8.3	8.2	8.2	8.2	8.2	8.3

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Table A3-1. (Continued) - pH (Standard Units)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	7.14	7.24	7.31	7.33	7.37	7.40	7.37
<u>Day 1</u>							
24 H	7.18	7.19	7.15	7.14	7.15	7.14	7.16
0 H	7.11	7.21	7.28	7.35	7.35	7.39	7.34
<u>Day 2</u>							
24 H	7.21	7.20	7.18	7.17	7.17	7.15	7.23
0 H	7.15	7.23	7.30	7.37	7.38	7.41	7.32
<u>Day 3</u>							
24 H	7.23	7.21	7.25	7.26	7.24	7.28	7.25
0 H	7.17	7.21	7.26	7.34	7.36	7.40	7.35
<u>Day 4</u>							
24 H	7.18	7.21	7.24	7.31	7.35	7.40	7.29
Min	7.11	7.19	7.15	7.14	7.15	7.14	7.16
Max	7.23	7.24	7.31	7.37	7.38	7.41	7.37

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Table A3-1. (Continued) - Temperature (°C)

	Test Concentrations (mg/L)						
	0.00	1.82	3.07	5.19	8.64	14.40	24.00
<u>Day 0</u>							
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 1</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 2</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 3</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
0 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
<u>Day 4</u>							
24 H	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Mean	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Min	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Max	24.0	24.0	24.0	24.0	24.0	24.0	24.0

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Table A3-2. FETAX Toxicity Test Data - Percent Embryo Mortality and Malformations after 96 Hours of Exposure to Chemical U2723 - Assay 3

Concentration (mg/L)	Rep	Number Embryos Alive	Percent Mortality	Number Embryos Malformed	Percent Malformed
Control	1	25	0.0	1	4.0
	2	25	0.0	1	4.0
	3	25	0.0	0	0.0
	4	25	0.0	0	0.0
1.82	1	25	0.0	1	4.0
	2	25	0.0	2	8.0
3.07	1	25	0.0	1	4.0
	2	25	0.0	1	4.0
5.19	1	25	0.0	1	4.0
	2	25	0.0	2	8.0
8.64	1	23	8.0	1	4.3
	2	20	20.0	5	25.0
14.40	1	14	44.0	4	28.6
	2	14	44.0	7	50.0
24.00	1	6	76.0	5	83.3
	2	5	80.0	3	60.0

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Table A3-3. FETAX Toxicity Test Data - Type and Number of Malformed Embryos after 96 Hours Exposure to Chemical U2723 - Assay 3

Malformation	Test Concentration (mg/L)															
	0 Rep				1.82 Rep				3.07 Rep				5.19 Rep			
	1	2	3	4	1	2	1	2	1	2	1	2	1	2	1	2
Severe																
Stunted																
Gut		1				2		1				1	3			2 3
Edema:																
Multiple												1				
Cardiac												1			3	
Abdominal												1				
Facial												1				
Cephalic																
Optic												1				
Tail																
Notochord						1					1		3	3		1
Fin																
Face	1				1	1	1		1	1			3	3	4	3 3
Eye																
Brain																
Hemorrhage																
Cardiac																2
Blisters																
Other*						1							3	1	3	1
# Malformed	1	1	0	0	1	2	1	1	1	2	1	5	4	7	5	3

* Anal edema.

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Table A3-4. FETAX Toxicity Test Data - Embryonic Growth (Length) after 96 Hours
Exposure to Chemical U2723 - Assay 3

Concentration (mg/L)	Rep	Number Embryos Alive	Average Length (mm)	Mean Average Length (mm)
Control	1	25	9.54	9.47
	2	25	9.64	
	3	25	9.31	
	4	25	9.40	
1.82	1	25	8.83	9.10
	2	25	9.36	
3.07	1	25	9.11	9.28
	2	25	9.44	
5.19	1	25	9.25	9.28
	2	25	9.30	
8.64	1	23	8.70	8.51*
	2	20	8.31	
14.40	1	14	8.08	8.11*
	2	14	8.14	
24.00	1	6	8.06	7.80*
	2	5	7.53	

* Significantly different at alpha = 0.05 (Bonferroni T-Test)

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APPENDIX 4

**ASSAY 1 RAW DATA SHEETS FOR FROG EMBRYO TERATOGENESIS
ASSAY-XENOPUS (FETAX) AND 6-AMINONICOTINAMIDE
REFERENCE TOXICANT FOR CHEMICAL U2723**

Note: The nominal concentrations of 1.9, 3.2, 5.4, 9.0, 15.0 and 25.0 mg/L in the raw data correspond to the nominal concentrations of 1.82, 3.07, 5.19, 8.64, 14.40 and 24.00 mg a.i./L in the report.

FORM 8D (Page 1 of 2): FETAX TOTAL SUMMARY DATA SHEET

 Notebook/Binder No. _____
 Page _____

Test Number Definite Test #1 Test Start Date 5/15/00 Test Material U2723
 Investigator S. D. Turkey Test End Date 5/17/00 Source _____
 Laboratory WAD-WREC Lot Number _____
 Concentration Steek - 25.0 mg/L
 Composition/Purity _____

Petri Dish Number	Concentration	Initial No. of Embryos	Total Mortality	% Mortality	Mean % Mortality	Total No. of Embryos Alive at 96 hrs.	Total No. Malformed	% Malformed	Mean % Malformed	Average Length (mm)	Mean Average Length (mm)
1	Control A	25	1	4	1	24	1	4.2	4.4	9.05	8.59
2	Control B	25	0	0		25	1	4.0		8.40	
3	Control C	25	0	0		25	1	4.0		8.29	
4	Control D	25	0	0		25	1	4.0		8.62	
5	1.9 mg/L A	25	1	4	2	24	2	8.3	8.2	8.44	8.29
6	1.9 mg/L B	25	0	0		25	2	8.0		8.14	
7	3.2 mg/L A	25	1	4	4	24	2	8.3	14.6	8.88	8.80
8	3.2 mg/L B	25	1	4		24	5	20.8		8.72	
9	5.4 mg/L A	25	2	8	10	23	7	30.4	22.0	8.42	8.51
10	5.4 mg/L B	25	3	12		22	3	13.6		8.60	
11	9.0 mg/L A	25	4	16	12	21	4	19.0	24.7	8.83	8.71
12	9.0 mg/L B	25	2	8		23	7	30.4		8.58	
13	15.0 mg/L A	25	9	36	38	16	9	56.3	61.8	8.41	8.08
14	15.0 mg/L B	25	10	40		15	11	73.3		7.74	
15	25.0 mg/L A	25	25	100	100	0					
16	25.0 mg/L B	25	25	100		0					
17											
18	6 Ammoniated										
19	5.5 mg/L A	25	2	8	10%	23	15	65.2	55.4	8.19	8.17
20	5.5 mg/L B	25	3	12		22	10	45.5		8.15	
21	2500 mg/L A	25	25	100	100	0					
22	2500 mg/L B	25	25	100		0					
23											
24											

 Prepared By S. D. Turkey Date 5/25/00

Reviewed By _____

Date _____

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Test Material U2723

[illegible]

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Petri Dish (Vial) No. 01 Test Number Definitive Test #1 Date Collected By Steve Turley
 Concentration FETAX Control A Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		23
MALFORMED			X																							1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut			X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face			X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Mal) No. 02 Test Number Definitive Test #1 Data Collected By Steve Turley No. Fixed Embryos 25

Concentration FETAX Control B Test Material U2723 Date 5/15-5/19/00

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED					X																					1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cul					X																					
Edema																										
Multiple																										
Cardiac					X																					
Abdominal																										
Facial																										
Cephalic																										
Opic																										
Tail																										
Notochord																										
Fin																										
Face					X																					
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 03 Test Number Definitive Test #1 Data Collected By Steve Turkey
 Concentration FETAX Control C Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 04 Test Number Definitive Test #1 Data Collected By Steve Turkey
Concentration FETAX Control D Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X																								
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 05 Test Number Definitive Test #1 Data Collected By Steve Turkey
Concentration 1.9 mg/L A Test Material U2723 Date 5/15-5/17/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		22
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face	X	X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

Reviewed by _____

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Petri Dish (Vial) No. 06 Test Number Definite Test #1 Data Collected By Steve Turkey
 Concentration 1.9 mg/L B Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X																								
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X	X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac		X																								
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 07 Test Number Definite Test 1 Data Collected By Steve Turley
Concentration 3.2 mg/L A Test Material 02723 Date 5/15-5/19/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		22
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Cul		X	X																							
Edema																										
Multiple																										
Cardiac			X																							
Abdominal			X																							
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face			X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 08 Test Number Definite Test 1 Data Collected By Steve Tuley
 Concentration 3.2 mg/L B Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		19
MALFORMED	X	X	X	X	X																					5
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X	X																					
Edema																										
Multiple			X		X																					
Cardiac			X	X	X																					
Abdominal	X		X		X																					
Facial			X		X																					
Cephalic																										
Optic			X		X																					
Tail																										
Notochord																										
Fin																										
Face			X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 09 Test Number Definitive Test 1 Data Collected By Steve Turley
Concentration 5.4 mg/L A Test Material 02723 Date 5/15/1-5/19/00 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE								X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			16
MALFORMED	X	X	X	X	X	X	X																			7
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X	X	X	X																			
Edema																										
Multiple	X				X																					
Cardiac					X																					
Abdominal					X																					
Facial	X				X																					
Cephalic																										
Optic	X				X																					
Tail																										
Notochord																										
Fin																										
Face	X	X	X			X																				
Eye																										
Brain																										
Hemorrhage																										
Cardiac		X	X																							
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 10 Test Number Definitive Test 1 Data Collected By Steve Turley
 Concentration 54 mg/L B Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				19
MALFORMED	X	X	X																							3
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face	X	X	X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 11 Test Number Definite Test 1 Data Collected By Steve Tedy
 Concentration 9.0 mg/L A Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 21

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					17
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut		X	X	X	X																					
Edema																										
Multiple		X	X																							
Cardiac		X	X																							
Abdominal		X	X																							
Facial		X	X																							
Cephalic																										
Optic			X																							
Tail																										
Notochord																										
Fin																										
Face			X	X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 12 Test Number Definite Test 1 Data Collected By Steve Turley
Concentration 9.0 mg/L B Test Material U2723 Date 5/15-5/19/60 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE								X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			16
MALFORMED	X	X	X	X	X	X	X																			7
MALFORMATIONS:																										
Severe	X																									
Stunted																										
Gut	X	X	X		X	X																				
Edema																										
Multiple	X			X																						
Cardiac	X			X																						
Abdominal	X																									
Facial	X			X																						
Cephalic																										
Optic			X																							
Tail																										
Notochord	X																									
Fin																										
Face	X	X					X																			
Eye	X																									
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 13 Test Number Definitive Test 1 Data Collected By Steve Turley
 Concentration 150 mg/L A Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 16

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE										X	X	X	X	X	X	X										7
MALFORMED	X	X	X	X	X	X	X	X	X																	9
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X			X		X	X																
Edema																										
Multiple							X	X	X	X																
Cardiac							X	X	X	X																
Abdominal							X	X	X																	
Facial							X	X	X	X																
Cephalic																										
Optic							X		X																	
Tail																										
Notochord								X																		
Fin																										
Face	X	X	X		X																					
Eye									X																	
Brain																										
Hemorrhage																										
Cardiac	X	X							X																	
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 14 Test Number Definitive Test 1 Data Collected By Steve Turkey
Concentration 15.0 mg/l B Test Material U2723 Date 5/15-5/19/00 No. Fixed Embryos 15

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE												X	X	X	X											4
MALFORMED	X	X	X	X	X	X	X	X	X	X	X															11
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X	X	X	X	X	X	X	X	X															
Edema																										
Multiple	X			X				X																		
Cardiac	X																									
Abdominal	X							X																		
Facial	X			X																						
Cephalic																										
Opik	X	X	X	X	X			X																		
Tail																										
Notochord																										
Fin																										
Face		X	X	X	X	X		X																		
Eye																										
Brain																										
Hemorrhage																										
Cardiac			X																							
Blisters																										
Other-Specify																										

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96 Hour Test Data

Effluent/Groundwater (Toxicant) Chemical U2723 Date: May 15-19, 2000

Definitive Test #1

Analyst: Steve TurleySpecies: Xenopus laevisAge at Start of Test: Black 6 (Stage 8-11)

Control	Day 0	Day 1		Day 2		Day 3		Day 4
FETAX Control	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.3	8.0	8.4	7.7	8.3	7.4	8.3	7.7
pH	7.74	7.26	7.24	7.16	7.22	7.11	7.13	7.11
Conductivity								
Alkalinity								
Hardness								

1.9 mg/L	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	7.8	8.4	7.7	8.3	7.5	8.2	7.7
pH	7.78	7.24	7.45	7.17	7.25	7.13	7.20	7.15
Conductivity								
Alkalinity								
Hardness								

3.2 mg/L	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	7.9	8.5	7.6	8.4	7.7	8.1	7.6
pH	7.79	7.30	7.38	7.20	7.28	7.16	7.23	7.18
Conductivity								
Alkalinity								
Hardness								

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Definite Test 1

96 Hour Test Data

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<u>5.4 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	7.9	8.4	7.7	8.3	7.7	8.2	7.5
pH	7.78	7.34	7.43	7.23	7.32	7.17	7.25	7.19
Conductivity								
Alkalinity								
Hardness								

<u>9.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	8.0	8.5	7.8	8.2	7.8	8.1	7.7
pH	7.78	7.35	7.43	7.23	7.34	7.25	7.26	7.18
Conductivity								
Alkalinity								
Hardness								

<u>15.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	8.0	8.5	7.8	8.3	7.9	8.1	7.7
pH	7.76	7.34	7.44	7.23	7.35	7.18	7.26	7.20
Conductivity								
Alkalinity								
Hardness								

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96 Hour Test Data

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<u>25.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.5	7.9	8.4	7.8	8.3	7.9	8.1	7.7
pH	7.62	7.33	7.32	7.25	7.27	6.95	7.18	7.19
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

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96 Hour Test Data

Effluent/Groundwater/Toxicant: 6-Am. monochloroamine (Test 1) Date: 5/15/50 - 5/19/50Analyst: STSpecies: Xeropus laevisAge at Start of Test: Day 8 - Stage 11

Control	Day 0	Day 1		Day 2		Day 3		Day 4
5.5 mg/L (6-AN)	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.4	8.0	8.3	7.9	8.3	7.8	8.3	7.6
pH	7.56	7.31	7.27	7.19	7.30	7.16	7.26	7.07
Conductivity								
Alkalinity								
Hardness								

2500 mg/L (6-AN)	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.3	7.9	8.3	7.7	8.3	7.6	8.3	7.4
pH	7.44	7.26	7.20	7.14	7.20	7.10	7.17	7.04
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

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Chemical U2723- Definitive Test 1
 Embryo Length Measurements (mm)
 Page 1 of x 3

	Control A	Control B	Control C	Control D	1.9 mg/L A	1.9 mg/L B
1	8.86	8.66	8.96	8.78	8.76	8.48
2	9.06	8.03	8.83	8.91	9	6.26
3	9.36	8.25	8.83	8.01	9.11	8.23
4	9.25	8.69	8.07	8.63	8.25	8.2
5	8.93	8.1	8.44	8.08	8.5	8.75
6	9.32	8.35	9.41	8.61	8.82	8.31
7	9.76	8.4	8.05	8.83	7.44	8.3
8	9.29	7.74	7.51	9.1	8.3	7.83
9	9.37	8.38	7.88	8.94	8.38	7.96
10	9.54	8.68	7.96	9	8.23	8.53
11	9.39	7.29	8.1	8.83	8.93	8.16
12	9.28	8.28	7.96	7.58	8.58	7.27
13	9.29	8.58	9.05	8.01	7.59	8.5
14	8.76	8.81	8.17	8.33	9.45	8.75
15	8.22	8.36	8.28	8.75	8.51	8.41
16	8.19	8.6	7.45	8.83	8.87	8.25
17	7.93	8.85	7.94	8.4	8.17	8.16
18	9.56	8.72	8.17	8.14	8.77	8.16
19	8.96	8.36	7.34	8.54	8.24	8.03
20	8.51	8.55	10.06	8.78	7.88	8.47
21	9.17	9.02	9.22	8.95	9.05	8.21
22	9.19	8.16	7.47	8.53	8.47	8.27
23	8.58	7.87	7.81	9.46	7.93	7.67
24	9.45	8.73	8.02	8.29	7.42	8.55
25		8.64	8.36	9.12		7.67
Mean	9.0508333	8.404	8.2936	8.6172	8.44375	8.1352
Min	7.93	7.29	7.34	7.58	7.42	6.26
Max	9.76	9.02	10.06	9.46	9.45	8.75
Stand. Dev.	0.4723477	0.3886194	0.6695949	0.429627	0.5323027	0.5190466

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Chemical U2723- Definitive Test 1
 Embryo Length Measurements (mm)
 Page 2 of 3

	3.2 mg/L A	3.2 mg/L B	5.4 mg/L A	5.4 mg/L B	9.0 mg/L A	9.0 mg/L B
1	9.24	9.22	5.61	9.7	8.73	9.16
2	9.15	8.66	8.41	8.92	8.89	8.52
3	8.84	9.4	6.85	7.33	7.24	9.18
4	7.53	8.78	6.63	9.14	8.4	6.34
5	8.95	8.74	8.59	8.98	8.37	8.6
6	9.21	8.76	7.42	8.25	8.44	8.84
7	8.01	9.09	8.98	8.45	9.51	8.89
8	9.25	8.34	8.73	8.37	9.23	7.41
9	9.54	9.37	8.92	6.25	8.66	8.83
10	9.39	8.44	8.6	8.84	8.87	8.73
11	8.07	8.35	8.39	8.75	6.98	9.26
12	10.08	8.03	7.61	9.26	8.32	9.45
13	9.2	9.04	8.51	7.97	8.83	9.42
14	8.84	9.18	9.35	8.79	9.15	9.22
15	8.94	8.76	8.16	8.5	9.5	8.99
16	8.09	8.07	9.1	8.93	9.45	9.01
17	9.38	7.17	9.41	9.11	9.81	9.82
18	8.78	8.97	8.72	9.76	9.09	9.4
19	8.08	9.18	8.91	8.72	8.94	6.95
20	9.66	9.58	9.32	8.33	9.6	7.57
21	8.84	8.27	9.57	8.58	9.52	8.24
22	9.28	8.61	8.79	8.27		9.24
23	7.69	8.23	9			6.33
24	9.16	9.04				
25						
Mean	8.8833333	8.72	8.4165217	8.6	8.8347619	8.5826087
Min	7.53	7.17	5.61	6.25	6.98	6.33
Max	10.08	9.58	9.57	9.76	9.81	9.82
Stand. Dev.	0.6501483	0.5448654	0.9830824	0.7544029	0.7235373	0.9891145

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Chemical U2723- Definitive Test 1
 Embryo Length Measurements (mm)
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	15 mg/L A		15 mg/L B		25 mg/L A		25 mg/L B		6-Aminonicotinamide	
	5.5 mg/L A		5.5 mg/L B		5.5 mg/L A		5.5 mg/L B		5.5 mg/L A	5.5 mg/L B
1		9		6.55					7.95	6.99
2		9.57		7.85					8.21	7.77
3		7.8		7.65					8.46	7.99
4		7.73		7.76					8.3	8.92
5		7.79		8.9					9.6	8.4
6		9.5		7.28					9.02	7.8
7		7.5		7.43					7.46	9.12
8		8.86		8.52					7.59	9.23
9		7.46		8.2					7.7	8.11
10		9.28		8.48					8.1	7.55
11		7.84		6.51					8.16	8.57
12		10.24							8.01	7.96
13		8.51							7.99	8.41
14		7.14							9.11	8.69
15		8.91							8.81	7.95
16		7.41							8.41	8.12
17									8.16	9.04
18									7.44	8.27
19									7.39	7.84
20									7.8	7.93
21									8.12	7.15
22									8.91	7.38
23									7.65	
24										
25										
Mean		8.40875		7.7390909					8.1891304	8.145
Min		7.14		6.51					7.39	6.99
Max		10.24		8.9					9.6	9.23
Stand. Dev.		0.9429519		0.7726895					0.5832504	0.6140556

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Petri Dish (Vial) No. 19 Test Number Definite Test 1 Data Collected By Steve Turley
 Concentration 5.5 mg/L A Test Material 6-Aminocaproic acid Date 5/15-5/19/77 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE																										8
MALFORMED	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	15
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X	X	X	X	X	X	X	X		X	X	X												
Edema																										
Multiple									X	X																
Cardiac								X	X	X				X												
Abdominal				X			X	X	X					X												
Facial								X																		
Cephalic																										
Optic																										
Tail												X	X			X										
Notochord	X		X								X	X														
Fin											X	X		X												
Face			X		X	X	X				X	X		X												
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters			X						X																	
Other-Specify																										

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Petri Dish (Vial) No. 20 Test Number Definite Test 1 Data Collected By Steve Turley
 Concentration 5.5 mg/L B Test Material 6-Aminocaproic acid Date 5/15-5/19/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE											X	X	X	X	X	X	X	X	X	X	X	X				12
MALFORMED	X	X	X	X	X	X	X	X	X	X																10
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut			X	X	X	X		X		X																
Edema																										
Multiple	X			X				X																		
Cardiac	X			X				X																		
Abdominal	X		X	X				X																		
Facial	X							X																		
Cephalic																										
Optic	X							X																		
Tail																										
Notochord		X					X			X																
Fin																										
Face		X			X	X	X		X	X																
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters							X																			
Other-Specify																										

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723 - Assay 1 Mortality - LC50

DATE: 5/15/00
TOXICANT : u2723
SPECIES: xenopus

TEST NUMBER: 1

DURATION: 96 h

RAW DATA:	Concentration	Number	Mortalities
---	-----	Exposed	
	.00	100	1
	2.00	50	1
	2.83	50	2
	4.73	50	5
	7.90	50	6
	14.70	50	19
	24.60	50	50

SPEARMAN-KARBER TRIM: 1.01%

SPEARMAN-KARBER ESTIMATES:	LC50:	13.77
95% LOWER CONFIDENCE:		12.38
95% UPPER CONFIDENCE:		15.31

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723-Assay 1 - Malformations (EC50)

DATE: 5/15/00
TOXICANT : U2723
SPECIES: xenopus

TEST NUMBER: 1

DURATION: 96 h

RAW DATA:	Concentration (mg/l)	Number Exposed	Mortalities
---	.00	99	4
---	2.00	49	4
---	2.83	48	7
---	4.73	45	10
---	7.90	44	11
---	14.70	31	20

SPEARMAN-KARBER TRIM: 36.98%

SPEARMAN-KARBER ESTIMATES:	EC50:	12.08
95% LOWER CONFIDENCE:		10.00
95% UPPER CONFIDENCE:		14.58

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

$$TI = LC50/EC50 = 13.77/12.08 = 1.14$$

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Title: u2723:Assay 1: embryo length data (mg)

File: ASSAY1LE.

Transform:

NO TRANSFORMATION

Shapiro - Wilk's Test for Normality

D = 0.6683

W = 0.9649

Critical W = 0.8250 (alpha = 0.01 , N = 14)

W = 0.8740 (alpha = 0.05 , N = 14)

Data PASS normality test (alpha = 0.01). Continue analysis.

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Title: u2723:Assay 1: embryo length data (mg)
File: ASSAY1LE. Transform: NO TRANSFORMATION

Bartlett's Test for Homogeneity of Variance

Calculated B1 statistic = 2.3953 (p-value = 0.7922)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Critical B = 15.0863 (alpha = 0.01, df = 5)
= 11.0705 (alpha = 0.05, df = 5)

Using Average Degrees of Freedom
(Based on average replicate size of 2.33)

Calculated B2 statistic = 2.2190 (p-value = 0.8181)

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

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Title: u2723:Assay 1: embryo length data (mg)

File: ASSAY1LE.

Transform:

NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.7451	0.1490	1.7838
Within (Error)	8	0.6683	0.0835	
Total	13	1.4134		

(p-value = 0.2224)

Critical F = 6.6318 (alpha = 0.01, df = 5,8)

= 3.6875 (alpha = 0.05, df = 5,8)

Since $F < \text{Critical } F$ FAIL TO REJECT H_0 : All equal (alpha = 0.05)

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Title: u2723:Assay 1: embryo length data (mg)

File: ASSAY1LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test

- TABLE 1 OF 2

Ho: Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG
1	con	8.5900	8.5900		
2	2.00 mg/l	8.2900	8.2900	1.1985	
3	2.83 mg/l	8.8000	8.8000	-0.8390	
4	4.73 mg/l	8.5100	8.5100	0.3196	
5	7.90 mg/l	8.7050	8.7050	-0.4594	
6	14.70 mg/l	8.0750	8.0750	2.0575	

Bonferroni t critical value = 2.8965 (1 Tailed, alpha = 0.05, df = 5,8)

Title: u2723:Assay 1: embryo length data (mg)

File: ASSAY1LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test

- TABLE 2 OF 2

Ho: Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	con	4			
2	2.00 mg/l	2	0.7250	8.4	0.3000
3	2.83 mg/l	2	0.7250	8.4	-0.2100
4	4.73 mg/l	2	0.7250	8.4	0.0800
5	7.90 mg/l	2	0.7250	8.4	-0.1150
6	14.70 mg/l	2	0.7250	8.4	0.5150

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APPENDIX 5

**ASSAY 2 RAW DATA SHEETS FOR FROG EMBRYO TERATOGENESIS
ASSAY-XENOPUS (FETAX) AND 6-AMINONICOTINAMIDE
REFERENCE TOXICANT FOR CHEMICAL U2723**

Note: The nominal concentrations of 1.9, 3.2, 5.4, 9.0, 15.0 and 25.0 mg/L in the raw data correspond to the nominal concentrations of 1.82, 3.07, 5.19, 8.64, 14.40 and 24.00 mg a.i./L in the report.

FORM 8D (Page 1 of 2): FETAX TOTAL SUMMARY DATA SHEET

Notebook Binder No. _____
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Test Number 2 Test Start Date 5/22/00 Test Material U2723 Concentration _____
 Investigator Joe T. Jey Test End Date 5/26/00 Source _____ Composition/Purity _____
 Laboratory UMD-WISER Lot Number _____

Petri Dish Number	Concentration	Initial No. of Embryos	Total Mortality	% Mortality	Mean % Mortality	Total No. of Embryos Alive at 96 hrs.	Total No. Malformed	% Malformed	Mean % Malformed	Average Length	Mean Average Length
1	FETAX Control A	25	0	0	1.0	25	1	4.0	4.1	8.94	8.88
2	FETAX Control B	25	0	0		25	2	8.0		8.81	
3	FETAX Control C	25	0	0		25	0	0		8.90	
4	FETAX Control D	25	1	4		24	1	4.2		8.88	
5	1.9 mg/L A	25	0	0	0	25	2	8.0	8.0	8.39	8.45
6	1.9 mg/L B	25	0	0		25	2	8.0		8.50	
7	3.2 mg/L A	25	2	8	10.0	23	2	8.7	4.4	8.42	8.50
8	3.2 mg/L B	25	3	12		22	0	0		8.71	
9	5.4 mg/L A	25	1	4	8.0	24	1	4.2	11.2	8.69	8.72
10	5.4 mg/L B	25	3	12		22	4	18.2		8.74	
11	9.0 mg/L A	25	3	12	10.0	22	4	18.2	20.0	7.94	7.93
12	9.0 mg/L B	25	2	8		23	5	21.7		7.92	
13	15.0 mg/L A	25	3	12	30.0	22	4	18.2	43.7	7.89	7.51
14	15.0 mg/L B	25	12	48		13	9	69.2		7.13	
15	25.0 mg/L A	25	16	64	70.0	9	6	66.7	66.7	7.50	7.39
16	25.0 mg/L B	25	19	76		6	4	66.7		7.27	
17											
18	6-Aminocaproic acid										
19	5.5 mg/L A	25	1	4	6.0	24	12	50.0	55.1	7.41	7.35
20	5.5 mg/L B	25	2	8		23	14	60.1		7.28	
21	250 mg/L A	25	25	100	100	—					
22	250 mg/L B	25	25	100		—					
23											
24											

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Reviewed By _____

Date _____

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Test Material V2723

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Petri Dish (Vial) No. 01 Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration FETAX Control A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Gul																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 02 Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration FETAX Control B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut			X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face			X	X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac			X	X																						
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 03 Test Number Definite Test 2 Data Collected By Stan Turkey
Concentration FETAX Control C Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	25
MALFORMED																										0
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 04 Test Number Definite Test 2 Data Collected By Steve Turkey
Concentration FETAX Control D Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		23
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord		X																								
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 05 Test Number Definitive Tot 2 Data Collected By Steve Turley
 Concentration 1.9 mg/L A Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut			X																							
Edema																										
Multiple		X	X																							
Cardiac		X	X																							
Abdominal		X	X																							
Facial		X	X																							
Cephalic																										
Optic		X	X																							
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 06 Test Number Definitive Tot 2 Data Collected By Steve Turley
 Concentration 1.9 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord			X																							
Fin																										
Face	X																									
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 07 Test Number Definite Test 2 Data Collected By Steve Turkey
Concentration 3.2 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				21
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord	X	X																								
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 08 Test Number Definitive Test 2 Data Collected By Steve Turley
 Concentration 3.2 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				22
MALFORMED																										0
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 09 Test Number Definite Test 2 Data Collected By Steve Turkey
 Concentration 5.4 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial		X																								
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 10 Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration 5.4 mg/L B Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				18
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X	X																						
Edema																										
Multiple	X	X																								
Cardiac	X	X																								
Abdominal	X	X	X																							
Facial	X	X	X																							
Cephalic																										
Optic	X																									
Tail																										
Notochord																										
Fin																										
Face		X	X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 11 Test Number Definitive Test 2 Data Collected By Steve Turley
 Concentration 9.0 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				18
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X																								
Edema																										
Multiple																										
Cardiac	X		X																							
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face	X			X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify			X																							

*Find edema

Reviewed By _____

Date _____

FORM 8H: FETAX INDIVIDUAL EMBRYO
MALFORMATION DATA

Notebook/Binder No. _____

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Petri Dish (Vial) No. 12 Test Number Definitive Test 2 Date Collected By Steve Turley
 Concentration 9.0 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			18
MALFORMED	X	X	X	X	X																					5
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X																							
Edema																										
Multiple	X																									
Cardiac	X																									
Abdominal	X																									
Facial	X																									
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face			X	X	X																					
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Mal) No. 013 Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration 15.0 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 22

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				18
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X	X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal				X																						
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord	X																									
Fin																										
Face			X																							
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 1A Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration 15.0 mg/L B Test Material U2723 Date 5/22/ - 5/26/00 No. Fixed Embryos 13

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE										X	X	X	X													4
MALFORMED	X	X	X	X	X	X	X	X	X																	9
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X	X	X				X	X	X																
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord	X																									
Fin																										
Face	X	X	X	X	X	X	X	X	X	X																
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify	X	X	X																							

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Petri Dish (Vial) No. 15 Test Number Definite Test 2 Data Collected By Steve Turley
 Concentration 250 mg/L A Test Material U2723 Date 5/22-5/26/03 No. Fixed Embryos 9

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE							X	X	X																	3
MALFORMED	X	X	X	X	X	X																				6
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X	X	X																				
Edema																										
Multiple	X		X	X																						
Cardiac	X		X	X		X																				
Abdominal	X		X	X																						
Facial	X		X	X																						
Cephalic																										
Optic	X		X	X																						
Tail																										
Notochord																										
Fin																										
Face					X																					
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 16 Test Number Definite Test 2 Data Collected By Steve Tinley
Concentration 25.0 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 6

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X																				2
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X																						
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X	X	X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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96 Hour Test Data

Effluent/Groundwater/Toxicant: Chemical U2723Date: 5/22-5/26/00Definitive Test 2Analyst: Steve TullySpecies: Xenopus laevisAge at Start of Test: Bufo (Stage 8-11)

Control	Day 0	Day 1		Day 2		Day 3		Day 4
FETAX	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.3	8.2	7.6	8.1	7.5	8.1	7.6
pH	7.14	7.12	7.11	7.18	7.15	7.20	7.17	7.11
Conductivity								
Alkalinity								
Hardness								

<u>1.9 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.3	8.2	7.8	8.1	7.6	8.2	7.6
pH	7.24	7.17	7.21	7.22	7.23	7.20	7.21	7.21
Conductivity								
Alkalinity								
Hardness								

<u>3.2 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.1	8.0	8.2	7.5	8.0	7.5	8.1	7.5
pH	7.31	7.21	7.28	7.23	7.30	7.22	7.26	7.20
Conductivity								
Alkalinity								
Hardness								

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Definitive Test 2

96 Hour Test Data

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<u>54 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.1	8.2	7.2	8.1	7.6	8.1	7.5
pH	7.33	7.23	7.35	7.24	7.37	7.25	7.34	7.29
Conductivity								
Alkalinity								
Hardness								

<u>9.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.1	8.1	8.1	7.2	8.0	7.7	8.0	7.6
pH	7.37	7.24	7.35	7.24	7.38	7.24	7.36	7.33
Conductivity								
Alkalinity								
Hardness								

<u>15.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.1	8.1	7.2	8.0	7.3	8.1	7.5
pH	7.40	7.26	7.39	7.23	7.41	7.23	7.40	7.39
Conductivity								
Alkalinity								
Hardness								

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Definitive Test 2

96 Hour Test Data

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<u>25.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
U2723	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.2	8.2	7.1	8.1	7.2	8.0	7.5
pH	7.37	7.27	7.34	7.24	7.32	7.25	7.35	7.34
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

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96 Hour Test Data

Effluent/Groundwater/Toxicant: 6-Aminocaproamide Date: 5/22-5/26/00Analyst: STSpecies: Xenopus laevisAge at Start of Test: 8-11

Control 5.5 mg/L	Day 0	Day 1		Day 2		Day 3		Day 4
(6-AN)	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.0	8.1	7.9	8.1	7.8	8.1	7.8
pH	7.10	7.08	7.15	7.11	7.17	7.06	7.13	7.02
Conductivity								
Alkalinity								
Hardness								

2500 mg/L	Day 0	Day 1		Day 2		Day 3		Day 4
(6-AN)	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	7.9	8.0	7.9	8.0	7.7	8.0	7.6
pH	7.03	7.00	7.08	7.07	7.14	7.03	7.07	6.98
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

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Chemical U2723- Definitive Test 2
 Embryo Length Measurements (mm)
 Page 1 of 3

	Control A	Control B	Control C	Control D	1.9 mg/L A	1.9 mg/L B
1	9.46	8.71	8.71	9.19	7.88	8.73
2	9.23	8.48	8.41	8.99	7.95	8.85
3	9.58	8.94	8.61	9.17	7.97	9.06
4	8.66	9.52	7.68	9.63	8.6	8.69
5	8.83	8.72	9.12	9.42	9.07	8.66
6	8.59	9.28	8.9	9.55	8.5	8.22
7	8.84	8.52	8.9	9.6	8.53	9.03
8	8.58	9.7	8.78	8.63	8.44	9.31
9	8.46	8.62	8.81	9.03	8.63	8.76
10	9.57	8.97	8.85	8.81	8.9	9.04
11	8.6	8.94	9.02	8.13	8.56	8.44
12	9.53	8.87	9.18	8.86	8.84	8.34
13	9.21	8.67	9.16	8.75	8.49	9.06
14	9.2	8.7	8.42	8.34	8.79	8.11
15	8.63	8.98	9.7	9.47	8.16	6.83
16	9.1	8.98	8.93	9	8.56	8.39
17	8.56	8.83	8.53	9.17	8.93	8.49
18	8.57	8.42	8.5	8.84	8.67	7.8
19	9.02	9.23	9.31	8.48	6.34	8.85
20	9.3	9.04	8.97	7.88	8.28	7.78
21	9.1	8.22	9.65	9.14	8.5	8.17
22	8.82	8.17	9.28	8.21	7.66	8.43
23	8.49	8.47	8.98	8.49	8.47	6.95
24	8.35	8.53	9.77	8.4	8.13	9.69
25	9.25	8.82	8.44		8.8	8.73
Mean	8.9412	8.8132	8.9044	8.8825	8.386	8.4964
Min	8.35	8.17	7.68	7.88	6.34	6.83
Max	9.58	9.7	9.77	9.63	9.07	9.69
Stand. Dev.	0.3858899	0.3679982	0.4612039	0.488211	0.553135	0.6562893

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Chemical U2723- Definitive 2
 Embryo Length Measurements (mm)
 Page 2 of 3

	3.2 mg/L A	3.2 mg/L B	5.4 mg/L A	5.4 mg/L B	9.0 mg/L A	9.0 mg/L B
1	7.7	8.54	8.73	7.03	7.4	7.3
2	8.98	9.23	8.42	9.07	7.64	8.85
3	8.48	9.17	9.03	9.01	8.25	8.39
4	7.95	8.54	8.88	9.04	7.93	8.09
5	9.32	9	8.65	9.81	7.99	8.11
6	8.98	9.09	8.65	8.53	8.19	7.13
7	8.51	8.42	8.42	8.25	7.48	8.48
8	8.13	8.31	7.96	8.95	7.34	7.83
9	8.73	8.61	8.98	8.28	7.73	8.23
10	8.73	8.66	9.24	8.48	7.32	7.97
11	7.66	8.27	9.14	8.21	7.55	8.19
12	7.86	9	8.39	9.26	7.33	7.31
13	8.38	8.12	8.69	9.14	8.11	8.06
14	8.32	8.35	8.26	8.18	7.92	7.51
15	8.3	8.47	8.53	8.55	8.21	8.36
16	8.24	8.04	8.69	7.79	8.3	7.47
17	8.32	8.93	9.32	9.68	8.38	9.67
18	8.54	8.45	8.62	9.65	7.79	8.3
19	8.06	8.52	8.74	9.22	8.87	7.37
20	8.61	9.24	8.03	8.86	8.35	7.98
21	7.65	9.18	7.94	8.3	8.65	7.04
22	9.63	9.47	9.22	9.07	7.97	7.8
23	8.58		9.46			6.73
24			8.52			
25						
Mean	8.42	8.7095455	8.6879167	8.7436364	7.9409091	7.9204348
Min	7.65	8.04	7.94	7.03	7.32	6.73
Max	9.63	9.47	9.46	9.81	8.87	9.67
Stand. Dev.	0.50842	0.4093139	0.4185792	0.6608259	0.4393167	0.6510933

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Chemical U2723- Definitive Test 2
 Embryo Length Measurements (mm)
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					6-Aminonicotinamide	
	15 mg/L A	15 mg/L B	25 mg/L A	25 mg/L B	5.5 mg/L A	5.5 mg/L B
1	7.5	7.58	7.91	6.84	7.34	7.02
2	9.29	6.28	7.54	7.67	7.89	7.16
3	8.34	7.34	7.59	7.13	6.68	6.8
4	8.99	6.88	8.18	7.31	8.06	6.91
5	8.86	6.66	7.37	7.37	7.73	6.99
6	7.98	7.03	6.64	7.29	7.26	7.16
7	8.31	6.77	6.93		7.59	7.24
8	8.23	7.96	7.67		6.88	7.33
9	7.73	7.86	7.69		7.4	7.09
10	7.08	6.73			7.13	7.77
11	7.48	7.84			7.21	7.91
12	7.77	7.19			7.44	8.09
13	7.96	6.53			7.07	7.5
14	7.99				7.67	7.55
15	7.75				7.14	7.35
16	6.78				6.98	7.22
17	7.34				7.49	7.61
18	7.59				7.31	7.42
19	7.22				7.3	6.8
20	7.5				8.1	6.76
21	7.51				7.12	7.14
22	8.39				7.57	7.37
23					7.38	7.26
24					8.1	
25						
Mean	7.8904545	7.1269231	7.5022222	7.2683333	7.41	7.2804348
Min	6.78	6.28	6.64	6.84	6.68	6.76
Max	9.29	7.96	8.18	7.67	8.1	8.09
Stand. Dev.	0.6280846	0.5508688	0.4725669	0.2744024	0.3776702	0.3482616

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Petri Dish (Vial) No. 19 Test Number Definitive Test 2 Data Collected By Steve Turley
 Concentration 5.5 mg/L Test Material 6-Aminocaproic acid Date 5/22 - 5/26/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE													X	X	X	X	X	X	X	X	X	X	X	X		12
MALFORMED	X	X	X	X	X	X	X	X	X	X	X	X														12
MALFORMATIONS:																										
Severe																										
Stunted																										
Cul	X	X			X	X	X	X	X		X	X														
Edema																										
Multiple						X																				
Cardiac				X		X																				
Abdominal						X		X				X														
Facial				X		X																				
Cephalic																										
Optic						X																				
Tail																										
Notochord			X			X		X	X	X	X															
Fin																										
Face		X	X		X						X															
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

Reviewed By _____

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Petri Dish (Vial) No. 20 Test Number Definitive Test 2 Data Collected By Steve Turkey
 Concentration 5.5 mg/L B Test Material 6-Aminocaproic acid Date 5/22-5/26/00 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE															X	X	X	X	X	X	X	X	X			9
MALFORMED	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X	X	X			X	X	X	X	X	X	X												
Edema												X														
Multiple				X	X							X														
Cardiac				X	X							X														
Abdominal		X		X	X			X				X														
Facial				X	X																					
Cephalic																										
Optic				X	X																					
Tail																										
Notochord					X	X	X	X				X		X												
Fin										X	X	X		X												
Face				X	X	X	X			X	X	X		X												
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723 - Assay 2 - Mortality (LC50)

DATE: 5/22/00
TOXICANT : U2723
SPECIES: XENOPUS

TEST NUMBER: 2

DURATION: 96 H

RAW DATA: Concentration --- ---- (MG/L)	Number Exposed	Mortalities
.00	100	1
1.91	50	0
3.04	50	5
4.82	50	4
7.97	50	5
13.30	50	15
23.10	50	35

SPEARMAN-KARBER TRIM: 30.20%

SPEARMAN-KARBER ESTIMATES: LC50: 17.61
95% LOWER CONFIDENCE: 15.53
95% UPPER CONFIDENCE: 19.96

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723-Assay 2-Malformations (EC50)

DATE: 5/22/00
TOXICANT : U2723
SPECIES: XENOPUS

TEST NUMBER: 2

DURATION: 96 H

RAW DATA:	Concentration	Number	Mortalities
---	(MG/L)	Exposed	
	.00	99	4
	1.91	50	4
	3.04	45	2
	4.82	46	5
	7.97	45	9
	13.30	35	13
	23.10	15	10

SPEARMAN-KARBER TRIM: 34.74%

SPEARMAN-KARBER ESTIMATES: EC50: 17.57
95% LOWER CONFIDENCE: 13.49
95% UPPER CONFIDENCE: 22.88

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

$$TI = LC50/EC50 = 1.00$$

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Title: u2723: embryo lengths- assay 2

File: ASSAY2LE.

Transform:

NO TRANSFORMATION

Shapiro - Wilk's Test for Normality

D = 0.3737

W = 0.9179

Critical W = 0.8440 (alpha = 0.01 , N = 16)

W = 0.8870 (alpha = 0.05 , N = 16)

Data PASS normality test (alpha = 0.01). Continue analysis.

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Title: u2723: embryo lengths- assay 2
File: ASSAY2LE. Transform: NO TRANSFORMATION

Bartlett's Test for Homogeneity of Variance

Calculated B1 statistic = 12.7727 (p-value = 0.0468)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Critical B = 16.8119 (alpha = 0.01, df = 6)
= 12.5916 (alpha = 0.05, df = 6)

Using Average Degrees of Freedom
(Based on average replicate size of 2.29)

Calculated B2 statistic = 9.8138 (p-value = 0.1327)

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

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Title: u2723: embryo lengths- assay 2

File: ASSAY2LE.

Transform:

NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	5.0788	0.8465	20.3873
Within (Error)	9	0.3737	0.0415	
Total	15	5.4525		

(p-value = 0.0001)

Critical F = 5.8018 (alpha = 0.01, df = 6,9)
= 3.3738 (alpha = 0.05, df = 6,9)

Since $F > \text{Critical } F$ REJECT H_0 : All equal (alpha = 0.05)

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Title: u2723: embryo lengths- assay 2

File: ASSAY2LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test		TABLE 1 OF 2		Ho: Control<Treatment	
GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	8.8825	8.8825		
2	1.91 mg/l	8.4450	8.4450	2.4793	
3	3.04 mg/l	8.5650	8.5650	1.7992	
4	4.82 mg/l	8.7150	8.7150	0.9492	
5	7.97 mg/l	7.9300	7.9300	5.3977	*
6	13.3 mg/l	7.5100	7.5100	7.7778	*
7	23.1 mg/l	7.3850	7.3850	8.4861	*

Bonferroni t critical value = 2.9333 (1 Tailed, alpha = 0.05, df = 6,9)

Title: u2723: embryo lengths- assay 2

File: ASSAY2LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test		TABLE 2 OF 2		Ho: Control<Treatment	
GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	4			
2	1.91 mg/l	2	0.5176	5.8	0.4375
3	3.04 mg/l	2	0.5176	5.8	0.3175
4	4.82 mg/l	2	0.5176	5.8	0.1675
5	7.97 mg/l	2	0.5176	5.8	0.9525
6	13.3 mg/l	2	0.5176	5.8	1.3725
7	23.1 mg/l	2	0.5176	5.8	1.4975

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APPENDIX 6

**ASSAY 3 RAW DATA SHEETS FOR FROG EMBRYO TERATOGENESIS
ASSAY-XENOPUS (FETAX) AND 6-AMINONICOTINAMIDE
REFERENCE TOXICANT FOR CHEMICAL U2723**

Note: The nominal concentrations of 1.9, 3.2, 5.4, 9.0, 15.0 and 25.0 mg/L in the raw data correspond to the nominal concentrations of 1.82, 3.07, 5.19, 8.64, 14.40 and 24.00 mg a.i./L in the report.

FORM 8D (Page 1 of 2): FETAX TOTAL SUMMARY DATA SHEET

Notebook/Binder No. _____
Page _____

Test Number 3 Test Start Date 5/22/00 Test Material U2723 Concentration _____
Investigator Steve Turley Test End Date 5/24/00 Source _____ Composition/Purity _____
Laboratory WMD/WRE Lot Number _____

Petri Dish Number	Concentration	Initial No. of Embryos	Total Mortality	% Mortality	Mean % Mortality	Total No. of Embryos Alive at 96 hrs.	Total No. Malformed	% Malformed	Mean % Malformed	Average Length	Mean Average Length
1	FETAX Control A	25	0	0	0	25	1	4.0	2.0	9.54	9.48
2	FETAX Control B	25	0	0		25	1	4.0		9.64	9.47ST
3	FETAX Control C	25	0	0		25	0	0		9.31	
4	FETAX Control D	25	0	0		25	0	0		9.40	
5	1.9mg/L A	25	0	0	0	25	1	4.0	6.0	8.83	9.10
6	1.9mg/L B	25	0	0		25	2	8.0		9.36	
7	3.2mg/L A	25	0	0	0	25	1	4.0	4.0	9.11	9.28
8	3.2mg/L B	25	0	0		25	1	4.0		9.44	
9	5.4mg/L A	25	0	0	0	25	1	4.0	6.0	9.25	9.28
10	5.4mg/L B	25	0	0		25	2	8.0		9.30	
11	9.0mg/L A	25	2	8	14	23	1	4.3	14.7	8.70	8.51
12	9.0mg/L B	25	5	20		20	5	25.0		8.31	
13	15.0mg/L A	25	11	44	44	14	4	28.6	39.3	8.08	8.11
14	15.0mg/L B	25	11	44		14	7	50.0		8.14	
15	25.0mg/L A	25	19	76	78	6	5	83.3	71.7	8.06	7.80
16	25.0mg/L B	25	20	80		5	3	60.0		7.53	
17	6-Aminocaproic acid										
18	5.5mg/L A	25	0	0	2.0	25	16	64.0	52.9	8.02	7.98
19	5.5mg/L B	25	1	4		24	10	41.7		7.93	
20	2500mg/L A	25	25	100	100	—					
21	2500mg/L B	25	25	100		—					
22											
23											

Prepared By _____ Date _____

Reviewed By _____

Date _____

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Notebook/Binder No.

Page

Test Material U2723

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Petri Dish (Vial) No. 01 Test Number Definite Test 3 Data Collected By Steve Turley
 Concentration FETAX Control A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cul																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 02 Test Number Daphnia Test 3 Data Collected By Steve Turley
 Concentration FETAX Control B Test Material U2723 Date 5/22-5/24/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
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Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 03 Test Number Definitive Test 3 Date Collected By Steve Turley
 Concentration FETAX Control Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	25
MALFORMED																										0
MALFORMATIONS:																										
Severe																										
Stunted																										
Cul																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 04 Test Number Desimbe Test 3 Data Collected By Steve Turley
 Concentration FETAX Control D Test Material U2723 Date 5/22-5/24/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	25
MALFORMED																										0
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
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Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 05 Test Number Definite Test 3 Data Collected By Steve Turley
 Concentration 19 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 06 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 19 mg/L B Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut		X	X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord			X																							
Fln																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify		X																								

*anal edema

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Petri Dish (Vial) No. 07 Test Number Definite Test 3 Data Collected By Steve Turley
 Concentration 3.2 mg/L A Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 08 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 3.2 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X																									
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
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Tail																										
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Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Mal) No. 09 Test Number Definitive Test 3 Date Collected By Steve Tuley
 Concentration 54 mg/L A Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	24
MALFORMED	X																									1
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 10 Test Number Desimbe Test 3 Data Collected By Steve Turley
 Concentration 54 mg/L B Test Material U2723 Date 5/22-5/24/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	23
MALFORMED	X	X																								2
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord		X																								
Fin																										
Face		X																								
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 11 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 9.0 mg/L A Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 23

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE		X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			22
MALFORMED	X																									
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X																									
Edema																										
Multiple	X																									
Cardiac	X																									
Abdominal	X																									
Facial	X																									
Cephalic																										
Optic	X																									
Tail																										
Notochord																										
Fin																										
Face																										
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

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Petri Dish (Vial) No. 12 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 90 mg/L B Test Material U2723 Date 5/22-5/26/00 No. Fixed Embryos 20

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X						15
MALFORMED	X	X	X	X	X																					5
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X		X	X																						
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Muscle/Chord	X	X				X																				
Fin																										
Face				X	X	X																				
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify *		X	X	X																						

* anal edema

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Petri Dish (Vial) No. 13 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 150 mg/L A Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 14

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE					X	X	X	X	X	X	X	X	X	X												10
MALFORMED	X	X	X	X																						4
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord	X	X		X																						
Fin																										
Face	X	X		X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisiers																										
Other-Specify *			X																							

* small edema

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Petri Dish (Vial) No. 14 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 15.0 mg/L B Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 14

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE								x	x	x	x	x	x	x												7
MALFORMED	x	x	x	x	x	x	x																			7
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut																										
Edema																										
Multiple																										
Cardiac				x	x		x																			
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face			x		x	x	x																			
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify	#	x				x		x																		

Reviewed By _____ Date _____
 Initial edema

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Petri Dish (Vial) No. 15 Test Number DeSoto Tit 3 Date Collected By Steve Turley
 Concentration 250 mg/L A Test Material 02723 Date 5/22-5/26/00 No. Fixed Embryos 6

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE						X																				5
MALFORMED	X	X	X	X	X																					
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut		X	X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord		X																								
Fin																										
Face			X		X	X																				
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify	X					X																				

and edema

Reviewed by _____

Date _____

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Petri Dish (Vial) No. 16 Test Number Definitive Test 3 Date Collected By Steve Turley
 Concentration 250 Test Material 02723 Date 5/22 - 5/24/00 No. Fixed Embryos 5

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE				X	X																					
MALFORMED	X	X	X																							
MALFORMATIONS:																										
Severe																										
Stunted																										
Gut	X	X	X																							
Edema																										
Multiple																										
Cardiac																										
Abdominal																										
Facial																										
Cephalic																										
Optic																										
Tail																										
Notochord																										
Fin																										
Face		X	X	X																						
Eye																										
Brain																										
Hemorrhage																										
Cardiac		X	X																							
Blisters																										
Other-Specify																										

Reviewed By _____

Date _____

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96 Hour Test Data

Effluent/Groundwater/Toxicant: Chemical 02723Date: 5/22-5/26/00Descriptive Test 3Analyst: Steve Tuckey

Species: _____

Age at Start of Test: 8 weeks (Stage 8-11)

Control	Day 0	Day 1		Day 2		Day 3		Day 4
FETAX SOLUTION	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.3	8.2	7.4	8.1	7.3	8.1	7.4
pH	7.14	7.18	7.11	7.21	7.15	7.23	7.17	7.18
Conductivity								
Alkalinity								
Hardness								

<u>1.9 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.3	8.2	7.2	8.1	7.1	8.2	7.5
pH	7.24	7.19	7.21	7.20	7.23	7.21	7.21	7.21
Conductivity								
Alkalinity								
Hardness								

<u>3.2 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.1	8.0	8.2	7.1	8.0	7.1	8.1	7.5
pH	7.31	7.15	7.28	7.18	7.30	7.25	7.26	7.24
Conductivity								
Alkalinity								
Hardness								

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Define Test 3

96 Hour Test Data

Page 2 of 3

<u>5.4 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.2	8.2	7.0	8.1	7.0	8.1	7.5
pH	7.33	7.14	7.35	7.17	7.37	7.26	7.34	7.31
Conductivity								
Alkalinity								
Hardness								

<u>9.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.1	8.2	8.1	7.0	8.0	7.0	8.0	7.6
pH	7.37	7.15	7.35	7.17	7.38	7.24	7.36	7.35
Conductivity								
Alkalinity								
Hardness								

<u>15.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	0 H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.2	8.1	7.0	8.0	7.1	8.1	7.4
pH	7.40	7.14	7.39	7.15	7.41	7.28	7.40	7.40
Conductivity								
Alkalinity								
Hardness								

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Definitive Test 3

96 Hour Test Data

Page 3 of 3

<u>25.0 mg/L</u>	Day 0	Day 1		Day 2		Day 3		Day 4
	O H	Old	New	Old	New	Old	New	Old
Temperature (°C)	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
D.O. (mg/L)	8.2	8.3	8.2	7.0	8.1	7.0	8.0	7.4
pH	7.37	7.16	7.34	7.23	7.32	7.25	7.35	7.29
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	O H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

	Day 0	Day 1		Day 2		Day 3		Day 4
	O H	Old	New	Old	New	Old	New	Old
Temperature (°C)								
D.O. (mg/L)								
pH								
Conductivity								
Alkalinity								
Hardness								

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Chemical U2723- Definitive Test 3
 Embryo Length Measurements (mm)
 Page 1 of 3

	Control A	Control B	Control C	Control D	1.9 mg/L A	1.9 mg/L B
1	10.02	10.08	9.74	9.45	9.14	9.37
2	9.75	9.88	9.05	9.06	9.31	9.48
3	9.76	10.03	9.31	9.65	9.68	9.88
4	9.85	9.9	8.93	8.4	8.86	9.78
5	9.47	9.22	8.85	9.46	8.54	9.13
6	9.59	9.59	9.09	8.59	9.57	8.11
7	9.06	9.48	8.87	9.64	8.3	8.7
8	9.38	9.88	8.92	9.1	8.5	9.44
9	9.32	9.28	8.54	9.42	8.25	9.28
10	9.67	9.88	9.62	9.49	8.94	9.42
11	9.7	9.09	9	8.74	8.79	9.41
12	9.71	9.84	9.44	9.8	9.01	7.68
13	9.64	9.04	9.79	8.73	8.62	8.68
14	9.5	9.88	9.91	9.73	9.03	8.97
15	9.59	9.38	9.54	9.28	9.14	9.13
16	9.44	9.99	8.47	9.25	8.28	10.1
17	9.88	9.46	9.83	9.16	8.49	9.37
18	9.38	9.74	9.38	9.32	7.82	9.42
19	9.76	9.51	9.82	9.8	8.8	9.24
20	8.98	9.52	10.02	9.17	7.93	10.01
21	9.37	9.67	9.22	10.21	9.26	10.22
22	9.38	9.52	9.58	9.94	8.93	9.87
23	9.65	10.23	9.53	9.85	9.52	9.55
24	9.59	9.15	9.54	9.68	9.11	9.65
25	8.95	9.8	8.76	10.09	8.83	10.14
Mean	9.5356	9.6416	9.31	9.4004	8.826	9.3612
Min	8.95	9.04	8.47	8.4	7.82	7.68
Max	10.02	10.23	10.02	10.21	9.68	10.22
Stand. Dev.	0.2697388	0.3274355	0.4414842	0.4640535	0.4844327	0.6033747

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Chemical U2723- Definitive Test 3
 Embryo Length Measurements (mm)
 Page 2 of 3

	3.2 mg/L A	3.2 mg/L B	5.4 mg/L A	5.4 mg/L B	9.0 mg/L A	9.0 mg/L B
1	9.34	9.49	9.75	9.71	9.44	8.17
2	8.47	9.37	9.09	9.33	8.31	8.61
3	9.15	9.79	9.15	8.93	8.4	8.29
4	9.36	9.47	8.73	8.62	9.1	8.12
5	8.48	9.61	9.29	8.58	8.8	8.22
6	9.48	9.72	9.53	9.37	8.71	8.27
7	7.61	9.44	9.02	9.29	7.97	8.87
8	9.9	9.18	9.69	9.89	9.08	7.86
9	9.26	8.94	9.36	8.66	8.31	9.38
10	9.24	9.4	9.28	9.71	6.15	8.33
11	8.91	8.75	9.38	9.37	8.43	7.21
12	8.58	10.02	9.52	9.3	9.33	7.68
13	9.57	9.82	9.01	9.53	9.24	8.31
14	9.59	9.47	9.02	8.98	9.17	9.25
15	9.05	9.37	9.12	9.55	9.03	7.78
16	9.15	9.17	10.11	8.9	9.14	7.76
17	9.4	8.81	8.9	9.02	8.79	7.04
18	8.59	8.81	9.12	9.23	8.48	8.93
19	9.44	9.8	8.25	9.66	9.3	8.66
20	9.42	9.81	8.85	8.37	8.97	9.36
21	9.46	9.83	8.24	9.7	9.07	
22	9.43	9.35	9.67	10	7.56	
23	9.47	10.01	9.93	10.03	9.35	
24	8.64	9.58	9.97	9.29		
25	8.73	9.06	9.28	9.46		
Mean	9.1088	9.4428	9.2504	9.2992	8.7013043	8.305
Min	7.61	8.75	8.24	8.37	6.15	7.04
Max	9.9	10.02	10.11	10.03	9.44	9.38
Stand. Dev.	0.5008852	0.3709281	0.4712526	0.4512291	0.7362331	0.6534483

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Chemical U2723- Definitive Test 3
 Embryo Length Measurements (mm)
 Page 3 of 3

				6-Aminonicotinamide		
	15 mg/L A	15 mg/L B	25 mg/L A	25 mg/L B	5.5 mg/L A	5.5 mg/L B
1	8.9	7.17	7.99	9.19	8.6	6.87
2	8.24	9.05	7.49	6.14	7.79	9.14
3	9.17	8.22	8.23	8	8.1	8.21
4	7.89	7.44	8.17	7.83	9.01	7.7
5	8.44	8.12	8.03	6.51	7.26	7.76
6	7.56	9.27	8.44		7.99	8.04
7	7.59	7.04			8.14	8.3
8	7.69	7.62			8.6	7.66
9	8.2	8.88			8.31	7.51
10	7.64	8.12			8.3	7.43
11	7.23	8.5			8.01	8.6
12	8.17	7.98			7.71	8.14
13	7.89	8.23			7.87	8.36
14	8.5	8.3			8.4	7.67
15					6.98	7.84
16					7.14	7.59
17					8.8	8.64
18					7.77	8.1
19					7.91	8.33
20					8.28	7.21
21					8.49	7.09
22					8.22	7.63
23					7.88	8.15
24					7.57	8.37
25					7.29	
Mean	8.0792857	8.1385714	8.0583333	7.534	8.0168	7.9308333
Min	7.23	7.04	7.49	6.14	6.98	6.87
Max	9.17	9.27	8.44	9.19	9.01	9.14
Stand. Dev.	0.5452941	0.6656386	0.3212735	1.2287107	0.5144959	0.5321974

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Petri Dish (Vial) No. 20 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 5.5 mg/L B Test Material 6-Aminocaproic acid Date 5/22-5/26/00 No. Fixed Embryos 24

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE											X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14
MALFORMED	X	X	X	X	X	X	X	X	X	X																10
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X		X	X	X	X	X	X																
Edema																										
Multiple			X						X																	
Cardiac			X																							
Abdominal			X					X	X																	
Facial			X					X																		
Cephalic																										
Optic			X																							
Tail																										
Notochord	X			X	X					X																
Fin																										
Face	X	X	X	X				X	X																	
Eye																										
Brain																										
Hemorrhage																										
Cardiac																										
Blisters																										
Other-Specify																										

Reviewed By _____

Date _____

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FORM 8H: FETAX INDIVIDUAL EMBRYO
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Petri Dish (Vial) No. 19 Test Number Definitive Test 3 Data Collected By Steve Turley
 Concentration 5.5 mg/L A Test Material 6-Aminocaproic acid Date 5/22 - 5/26/00 No. Fixed Embryos 25

EMBRYO NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	TOTAL
NOT REMARKABLE																	X	X	X	X	X	X	X	X	X	9
MALFORMED	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X										16
MALFORMATIONS:																										
Severe																										
Stunted																										
Cut	X	X	X	X	X			X	X	X	X	X	X	X	X											
Edema																										
Multiple												X	X	X												
Cardiac												X	X	X	X											
Abdominal			X	X				X			X	X	X	X												
Facial												X	X	X												
Cephalic												X	X													
Optic												X	X													
Tail																										
Notochord		X			X			X																		
Fin												X	X	X		X										
Face				X	X	X																				
Eye																										
Brain																										
Hemorrhage																										
Cardiac													X													
Blisters																										
Other-Specify																										

Reviewed By _____

Date _____

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723-Assay 3-Mortality (LC50)

DATE: 5/22/00
TOXICANT : u2723
SPECIES: xenopus

TEST NUMBER: 3

DURATION: 96 h

RAW DATA:	Concentration	Number	Mortalities
---	-----	Exposed	
	(mg/l)		
	.00	100	0
	1.93	50	0
	3.27	50	0
	5.25	50	0
	8.26	50	7
	14.00	50	22
	23.90	50	39

SPEARMAN-KARBER TRIM: 22.00%

SPEARMAN-KARBER ESTIMATES:	LC50:	15.26
	95% LOWER CONFIDENCE:	13.13
	95% UPPER CONFIDENCE:	17.75

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TRIMMED SPEARMAN-KARBER METHOD. VERSION 1.5

U2723 - Assay 3 - Malformations (EC50)

DATE: 5/22/00
TOXICANT : U2723
SPECIES: XENOPUS

TEST NUMBER: 3

DURATION: 96 H

RAW DATA: Concentration --- ---- (MG/L)	Number Exposed	Mortalities
.00	100	2
1.93	50	3
3.27	50	2
5.25	50	3
8.26	43	6
14.00	28	11
23.90	11	8

SPEARMAN-KARBER TRIM: 27.83%

SPEARMAN-KARBER ESTIMATES: EC50: 16.79
95% LOWER CONFIDENCE: 12.35
95% UPPER CONFIDENCE: 22.82

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

TI - LC50/EC50 = 0.91

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Title: U2723:ASSAY 3: embryo lengths (mg)

File: ASSAY3LE.

Transform:

NO TRANSFORMATION

Shapiro - Wilk's Test for Normality

D = 0.5007

W = 0.9545

Critical W = 0.8440 (alpha = 0.01 , N = 16)

W = 0.8870 (alpha = 0.05 , N = 16)

Data PASS normality test (alpha = 0.01). Continue analysis.

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Title: U2723:ASSAY 3: embryo lengths (mg)
File: ASSAY3LE. Transform: NO TRANSFORMATION

Bartlett's Test for Homogeneity of Variance-----
Calculated B1 statistic = 5.7971 (p-value = 0.4463)Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Critical B = 16.8119 (alpha = 0.01, df = 6)
= 12.5916 (alpha = 0.05, df = 6)

Using Average Degrees of Freedom
(Based on average replicate size of 2.29)

Calculated B2 statistic = 5.0874 (p-value = 0.5327)

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

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Title: U2723:ASSAY 3: embryo lengths (mg)

File: ASSAY3LE.

Transform:

NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	6	5.9602	0.9934	17.8546
Within (Error)	9	0.5007	0.0556	
Total	15	6.4609		

(p-value = 0.0002)

Critical F = 5.8018 (alpha = 0.01, df = 6,9)

= 3.3738 (alpha = 0.05, df = 6,9)

Since $F > \text{Critical } F$ REJECT H_0 : All equal (alpha = 0.05)

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Title: U2723:ASSAY 3: embryo lengths (mg)

File: ASSAY3LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test		TABLE 1 OF 2		Ho: Control<Treatment	
GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	CONTROL	9.4725	9.4725		
2	1.93 mg/l	9.1150	9.1150	1.7501	
3	3.27 mg/l	9.2750	9.2750	0.9668	
4	5.25 mg/l	9.2750	9.2750	0.9668	
5	8.26 mg/l	8.5050	8.5050	4.7363	*
6	14.0 mg/l	8.1100	8.1100	6.6700	*
7	23.9 mg/l	7.7950	7.7950	8.2121	*

Bonferroni t critical value = 2.9333 (1 Tailed, alpha = 0.05, df = 6,9)

Title: U2723:ASSAY 3: embryo lengths (mg)

File: ASSAY3LE.

Transform:

NO TRANSFORMATION

Bonferroni t-Test		TABLE 2 OF 2		Ho: Control<Treatment	
GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	CONTROL	4			
2	1.93 mg/l	2	0.5992	6.3	0.3575
3	3.27 mg/l	2	0.5992	6.3	0.1975
4	5.25 mg/l	2	0.5992	6.3	0.1975
5	8.26 mg/l	2	0.5992	6.3	0.9675
6	14.0 mg/l	2	0.5992	6.3	1.3625
7	23.9 mg/l	2	0.5992	6.3	1.6775

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ATTACHMENT 1

PHOTOGRAPHS OF NORMAL AND MALFORMED EMBRYOS EXPOSED TO
CHEMICAL U2723

<u>Photo:</u>	<u>Description:</u>
1.	Assay 1; FETAX solution exposure
2.	Assay 1; FETAX solution exposure
3.	Assay 1; 8.64 mg a.i./L (nominal concentration) exposure
4.	Assay 1; 8.64 mg a.i./L (nominal concentration) exposure
5.	Assay 2; 5.19 mg a.i./L (nominal concentration) exposure
6.	Assay 2; 5.19 mg a.i./L (nominal concentration) exposure
7.	Assay 2; 24.0 mg a.i./L (nominal concentration) exposure
8.	Assay 2; 24.0 mg a.i./L (nominal concentration) exposure
9.	Assay 3; 14.4 mg a.i./L (nominal concentration) exposure
10.	Assay 3; 14.4 mg a.i./L (nominal concentration) exposure
11.	Assay 1; 8.64 mg a.i./L (nominal concentration) exposure
12.	Assay 1; 8.64 mg a.i./L (nominal concentration) exposure
13.	Assay 1; 14.4 mg a.i./L (nominal concentration) exposure
14.	Assay 1; 8.64 mg a.i./L (nominal concentration) exposure

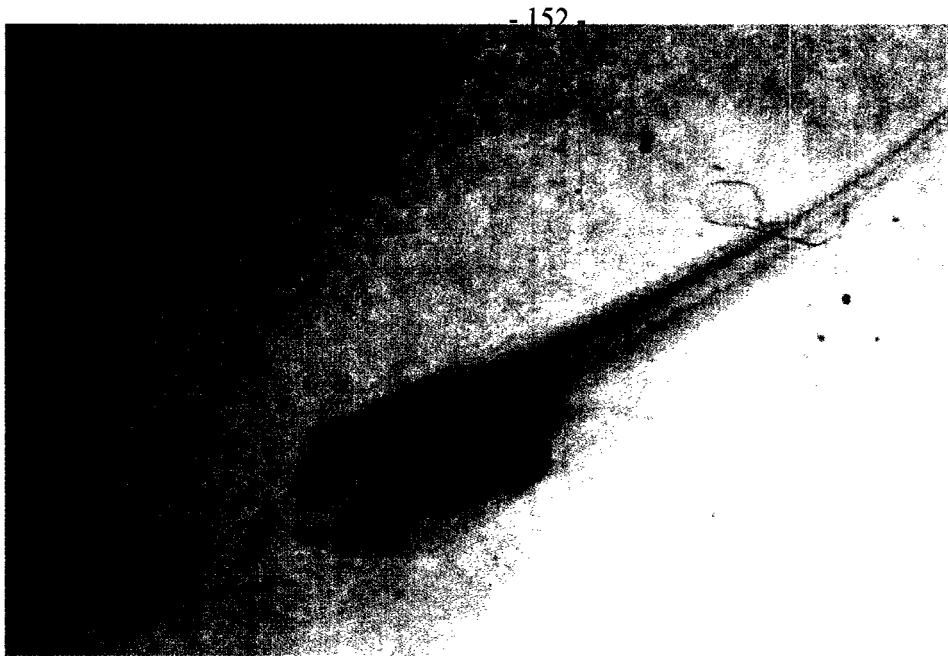


Photo 1: (7.5x) Normally developing *Xenopus laevis* embryo at 96 hours. Note- tight coiling of the gut and full head region.

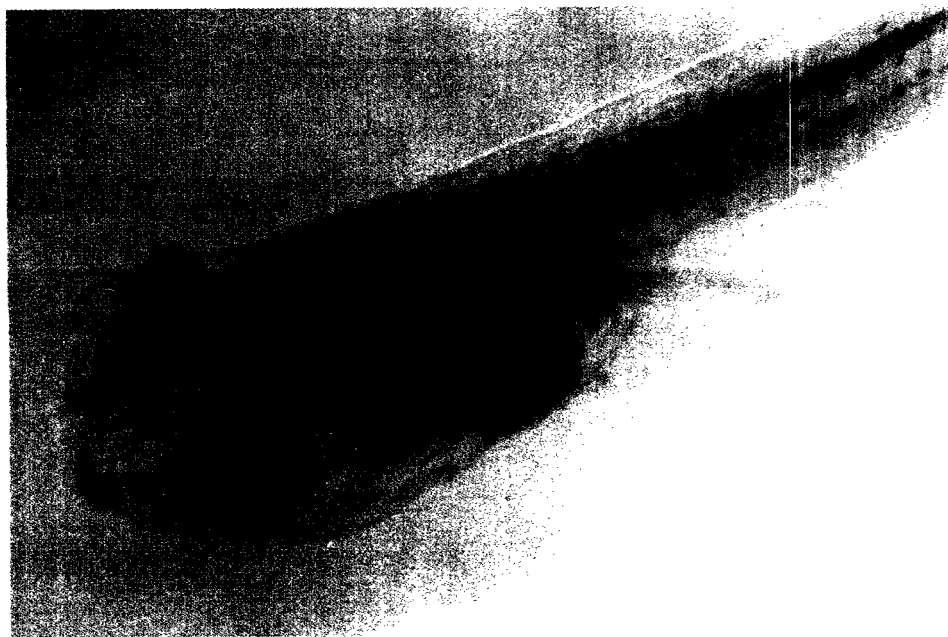


Photo 2: (15x) Normally developing *Xenopus laevis* embryo at 96 hours.

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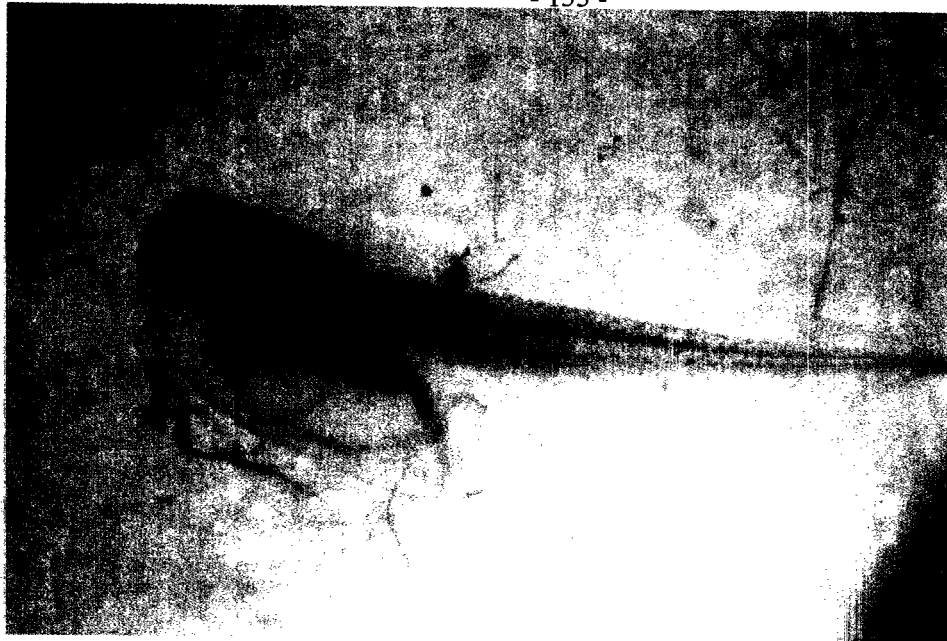


Photo 3: (7.5x) Malformations observed: Multiple edemas, including gut, cardiac, facial and optic edemas; facial abnormalities and improper gut coiling.



Photo 4: (15x) Malformations observed: Multiple edemas, including gut, cardiac, facial and optic edemas; facial abnormalities and improper gut coiling.

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Photo 5: (15x) Malformation observed: Improper (loose) gut coiling.



Photo 6: (15x) Malformation observed: Improper (loose) gut coiling.

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Photo 7: (7.5x) Malformations observed: Multiple edemas, including gut, cardiac, facial and optic edemas; extreme lack of gut coiling; severe facial abnormalities.



Photo 8: (15x) Malformations observed: Multiple edemas, including gut, cardiac, facial and optic edemas; extreme lack of gut coiling; severe facial abnormalities.

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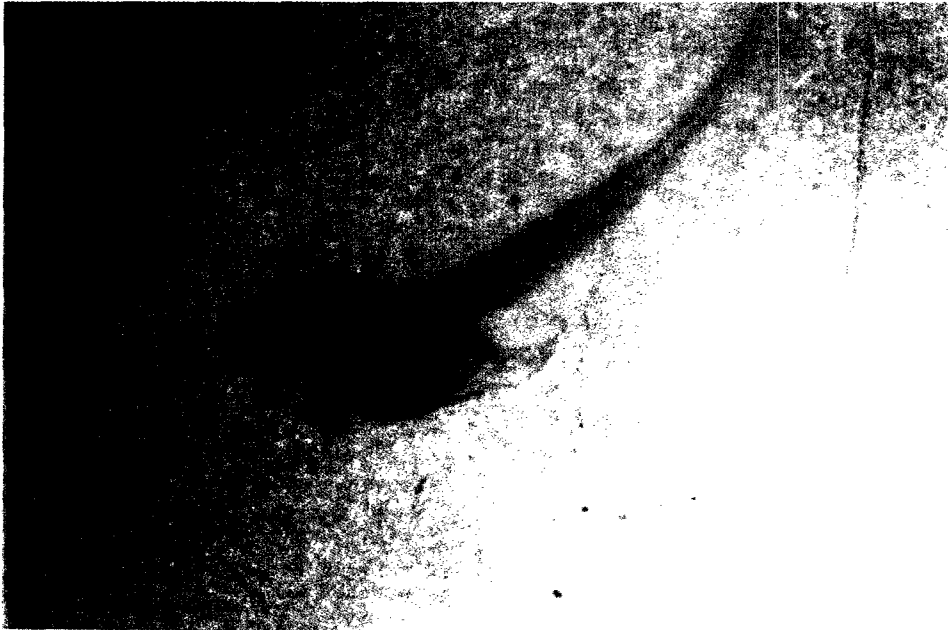


Photo 9: (7.5x): Malformations observed: Anal edema; slight facial abnormalities; slightly curved notochord.

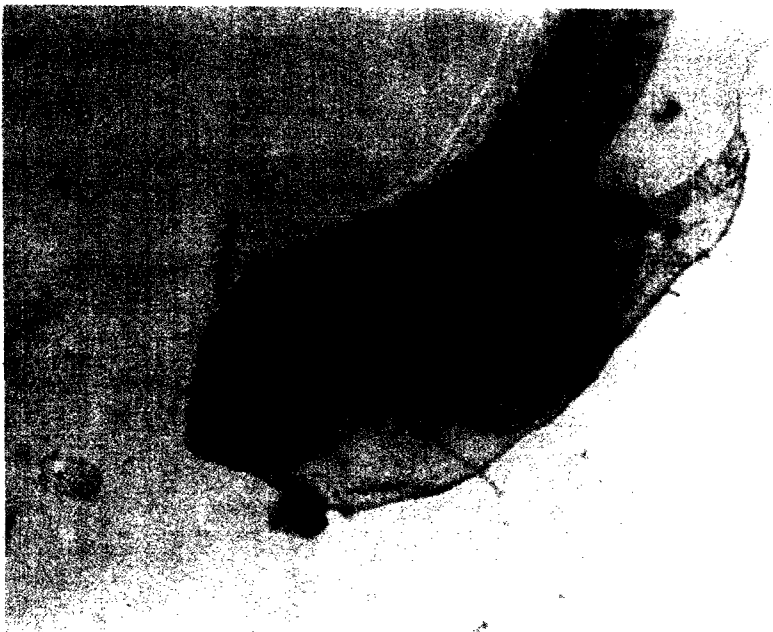


Photo 10: (15x): Malformations observed: Anal edema; slight facial abnormalities; slightly curved notochord.

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Picture 11: (7.5x) Malformations observed: Severe cardiac edema; slight gut edema; extremely improper gut coiling, curvature of the notochord; slight facial abnormality (caused by cardiac edema).

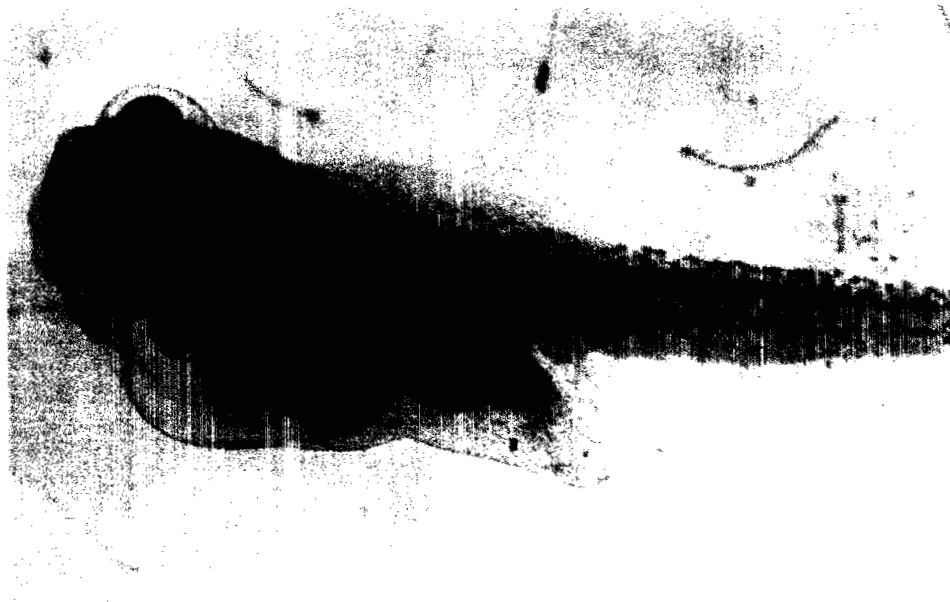


Picture 12: (15x) Malformations observed: Severe cardiac edema; slight gut edema; extremely improper gut coiling; curvature of the notochord; slight facial abnormality (caused by cardiac edema).

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Picture 13: (15x) Malformations observed: Multiple edemas, including cardiac, gut, facial and optic edemas; severe facial abnormalities; severe lack of gut coiling.



Picture 14: (15x) Malformations observed: Multiple edemas, including cardiac, gut, facial and optic edemas; severe facial abnormalities; severe lack of gut coiling.

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ATTACHMENT 2

LETTER FROM STEVEN TURLEY OF
UNIVERSITY OF MARYLAND, WYE RESEARCH AND EDUCATION CENTER

March 19, 2001

Mrs. Sue Palmer
Wildlife International, Ltd.
8598 Commerce Drive
Easton, MD 21601

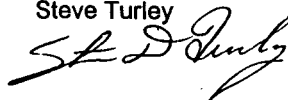
Dear Sue:

Enclosed is a copy of the revised final report. All of the suggested questions or corrections were addressed. Although LC50, EC50 and MCIG values changed when actual mean measured concentrations were used, the TI values were the same for all three tests. Thus, data still suggest that this chemical has little teratogenic potential based on FETAX results.

On the reference toxicant question, the 2500 mg/L concentration has consistently caused high mortality at our laboratory. The low reference toxicant concentration has also been very consistent, with low mortality and approximately 50% malformed embryos. I feel that the mortality observed in the high reference toxicant concentration does not compromise the results of these three assays in any way. Other laboratories working with FETAX often have similar high mortality in the 2,500 mg/L concentration. Over the last 5 years our laboratory has performed numerous standard and modified FETAX assays. In each study, there has been consistently high mortality in the 2,500 mg/L reference toxicant exposure, while mortality and malformations have been extremely low in embryos reared in FETAX solution. The best example of this was an extended FETAX study performed last year for the U.S. Fish and Wildlife Service. At the 2,500 mg/L reference toxicant concentration, there was 100% mortality. In the FETAX solution exposures, greater than 90% of the embryos survived (and completed metamorphosis) 140-d post-hatch. I think this example demonstrates the quality of embryos produced by our in-house culture.

The lack of a signature in the review space was an oversight on my part. The data was reviewed initially by our former QAQC person at the lab. Since she was no longer a WREC employee, she did not know whether she could legally sign the data sheets. This employee was my wife (and believe me- there are no harsher critics than a spouse). After she reviewed the data, Dr. Burton and I reviewed the data again. The lack of initials was just an oversight on my part. After receiving the report from you for the revisions, I reviewed the data again and found no mistakes. Please review the revised report, and if you have any corrections let me know. I will make any changes you deem necessary.

Sincerely,
Steve Turley



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APPENDIX III

The Analysis of PFOS in Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)
Solutions in Support of Wildlife International, Ltd. Project No.: 454A-116

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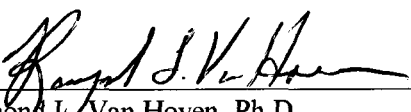
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-116

PRINCIPAL INVESTIGATOR:



Raymond L. Van Hoven, Ph.D.
Scientist

4/6/01

DATE

MANAGEMENT:

 for Will Nixon

Willard B. Nixon, Ph.D.
Director, Analytical Chemistry

4/6/01

DATE

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Introduction

Frog embryo teratogenesis assay-*Xenopus* (FETAX) solution samples were collected from three definitive assays designed to determine the effects of PFOS (Perfluorooctanesulfonate, Potassium Salt) to the embryos of the South African clawed frog (*Xenopus laevis*). This study was conducted by Wildlife International, Ltd. and identified as Project No.: 454A-116. The analyses of these FETAX solution samples were performed at Wildlife International, Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Test solutions for the first assay were prepared on May 15, 2000 and samples from these solutions were analyzed on May 15 and 22, 2000. Test solutions for the second and third assay were prepared on May 19, 2000 and samples from these solutions were analyzed on May 19 and 30, 2000.

Test Substance and Internal Standard

The test substance used for this study was Wildlife International, Ltd. identification number 4675. The test substance was used to prepare calibration and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International, Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard (hereafter referred to as 4HPFOS) was stored under ambient conditions.

Analytical Method

The method used for the analysis of the FETAX solution samples was based on methodology developed at Wildlife International, Ltd. and entitled "Analytical Method for the Determination of PFOS in Freshwater, Saltwater, and Algal Medium". This methodology was included as Appendix II of Wildlife International, Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation.

Samples were diluted in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 3000 Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C₁₈ analytical column (50 mm × 2 mm I.D., 3-μm particle size). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure[®] water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.00480 to 0.0480 mg a.i./L, were analyzed with the samples. The same and most prominent peak response for PFOS was utilized to monitor PFOS in all calibration, quality control, and study samples. No attempt was made to quantify PFOS on the basis of individual isomeric components. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentration ratios (PFOS : internal standard) of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.240 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00480 mg a.i./L) and the dilution factor of the matrix blank samples (50).

Matrix Blank and Fortification Samples

Four matrix blank samples were analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). A representative ion chromatogram of a matrix blank is presented in Figure 5.

FETAX solution was fortified at 0.480, 6.72 and 28.8 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected

for the mean procedural recovery of 97.2%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-116-7, nominal concentration of 24.0 mg a.i./L in FETAX solution.

Peak Area Ratio = Analyte Peak Area/Internal Standard Peak Area

Concentration Ratio = Concentration of Analyte/Concentration of Internal Standard

Internal Standard Concentration: 0.100 mg a.i./L

Initial Volume: 0.100 mL

Final Volume: 100 mL

Dilution Factor: 1000

PFOS Peak Area: 1158987

Internal Standard Peak Area: 2125037

Peak Area Ratio: 0.5454

Calibration curve equation.

Slope: 1.9275

Intercept: 0.0260

Curve is weighted (1/x)

$$\begin{aligned}\text{PFOS (mg a.i./L) at Instrument} &= \frac{\text{Peak area ratio} - (\text{Y-intercept})}{\text{Slope}} \times \text{Internal Standard Concentration} \\ &= \frac{0.5454 - 0.0260}{1.9275} \times 0.100 \\ &= 0.02695\end{aligned}$$

$$\begin{aligned}\text{PFOS (mg a.i./L) in sample} &= \text{PFOS (mg a.i./L) at Instrument} \times \text{Dilution Factor} \\ &= 0.02695 \times 1000 \\ &= 26.95\end{aligned}$$

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$$\begin{aligned}\text{Percent of Nominal Concentration} &= \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100 \\ &= \frac{26.95}{24.0} \times 100 \\ &= 112\%\end{aligned}$$

RESULTS

Sample Analysis

FETAX solution samples were collected from each of three definitive FETAX assays prior to test initiation (Day 0) and at test termination (Day 4 or 96 hours). The first assay was initiated on May 15, 2000 and terminated on May 19, 2000. The second and third assays were initiated on May 19, 2000 and terminated on May 23, 2000.

In the first assay, the measured concentrations of PFOS in test solution samples collected prior to initiation of exposure of the test organisms ranged from 112 to 141% of the nominal concentrations. Samples collected at test termination had a measured concentration range of 54.7 to 98.6% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present (Table 4). A representative ion chromatogram of a test sample is shown in Figure 7.

For the second and third assays, common test solutions were prepared. The measured concentrations of PFOS in test solution samples collected prior to initiation of exposure of the test organisms ranged from 95.8 to 117% of the nominal concentrations (Tables 5 and 6).

In the second assay, samples collected at test termination had a measured concentration range of 80.7 to 112% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present (Table 5).

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In the third assay, samples collected at test termination had a measured concentration range of 93.4 to 114% of nominal values. The sample from the abiotic 24.0 mg a.i./L treatment group was comparable to samples from the 24.0 mg a.i./L treatment group with the frog embryos present (Table 6).

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Table 1

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 3000 Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (50 mm × 2 mm I.D., 3-μm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	5.00 minutes
FLOW RATE:	220 μL/minute
MOBILE PHASE:	70.0% Methanol : 30.0% NANOpure [®] Water containing 0.1% Formic Acid
INJECTION VOLUME:	5.0 μL
PFOS RETENTION TIME:	Approximately 3.2 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 2.1 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

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Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Sample Set	Measured Concentration of PFOS ¹ (mg a.i./L)
Number (454A-116-)	Type		
MAB-1	Matrix Blank	Day 0 - 1 st Assay	< LOQ
MAB-2	Matrix Blank	Day 0 - 2 nd and 3 rd Assay	< LOQ
MAB-3	Matrix Blank	Day 4 - 1 st Assay	< LOQ
MAB-4	Matrix Blank	Day 4 - 2 nd and 3 rd Assay	< LOQ

¹ The limit of quantitation (LOQ) was 0.240 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00480 mg a.i./L) and the dilution factor of the matrix blank samples (50).

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Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-116-)	Sample Set	Concentrations of PFOS (mg a.i./L)		Percent Recovered ²
		Fortified ¹	Measured ¹	
MAS-1	Day 0 - 1 st Assay	0.480	0.490	102
MAS-2		6.72	7.12	106
MAS-3		28.8	30.2	105
MAS-4	Day 0 - 2 nd and 3 rd Assay	0.480	0.441	91.8
MAS-5		6.72	6.32	94.0
MAS-6		28.8	27.4	95.1
MAS-7	Day 4 - 1 st Assay	0.480	0.443	92.3
MAS-8		6.72	6.20	92.2
MAS-9		28.8	27.7	96.1
MAS-10	Day 4 - 2 nd and 3 rd Assay	0.480	0.449	93.5
MAS-11		6.72	6.77	101
MAS-12		28.8	28.1	97.6
Mean =				97.2
Standard Deviation =				5.08
CV =				5.22%
N =				12

¹ Concentrations were corrected for change in test substance purity (90.49% to 86.9%) per Certificate of Analysis dated September 7, 2000.

² Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since fortified and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

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Table 4

Measured Concentrations of PFOS in Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)
Solution Samples - First Assay

Nominal Test Concentration ¹ (mg a.i./L)	Sample Number (454A-116-)	Sampling Time (Day)	PFOS Measured Concentration ^{1,2} (mg a.i./L)	Percent of Nominal ³
0.0 (Negative Control)	1 8	0 4	< LOQ < LOQ	-- --
0.0 (Positive Control)	9	4	< LOQ	--
1.82	2 10	0 4	2.58 1.42	141 77.8
3.07	3 11	0 4	3.94 1.72	128 55.9
5.19	4 12	0 4	6.62 2.84	128 54.7
8.64	5 13	0 4	10.7 5.09	124 58.9
14.4	6 14	0 4	18.5 10.8	128 74.9
24.0	7 15	0 4	26.9 22.3	112 93.0
24.0 (Abiotic)	16	4	23.7	98.6

¹ Concentrations were corrected for change in test substance purity (90.49% to 86.9%) per Certificate of Analysis dated September 7, 2000.

² The limit of quantitation (LOQ) was 0.240 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00480 mg a.i./L) and the dilution factor of the matrix blank samples (50).

³ Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since fortified and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

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Table 5

Measured Concentrations of PFOS in Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)
Solution Samples - Second Assay

Nominal Test Concentration ¹ (mg a.i./L)	Sample Number (454A-116-)	Sampling Time (Day)	PFOS Measured Concentration ^{1,2} (mg a.i./L)	Percent of Nominal ³
0.0 (Negative Control)	17	0	< LOQ	--
	24	4	< LOQ	--
1.82	18	0	1.77	97.3
	26	4	2.04	112
3.07	19	0	3.59	117
	27	4	2.49	81.0
5.19	20	0	5.45	105
	28	4	4.18	80.7
8.64	21	0	8.43	97.6
	29	4	7.51	86.9
14.4	22	0	14.5	100
	30	4	12.1	84.3
24.0	23	0	23.0	95.8
	31	4	23.1	96.4
24.0 (Abiotic Control)	32	4	23.9	99.6

¹ Concentrations were corrected for change in test substance purity (90.49% to 86.9%) per Certificate of Analysis dated September 7, 2000.

² The limit of quantitation (LOQ) was 0.240 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00480 mg a.i./L) and the dilution factor of the matrix blank samples (50).

³ Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since fortified and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

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Table 6

Measured Concentrations of PFOS in Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)
Solution Samples - Third Assay

Nominal Test Concentration ¹ (mg a.i./L)	Sample Number (454A-116-)	Sampling Time (Day)	PFOS Measured Concentration ^{1,2} (mg a.i./L)	Percent of Nominal ³
0.0 (Negative Control)	17	0	< LOQ	--
	33	4	< LOQ	--
1.82	18	0	1.77	97.3
	35	4	2.08	114
3.07	19	0	3.59	117
	36	4	2.94	95.7
5.19	20	0	5.45	105
	37	4	5.05	97.5
8.64	21	0	8.43	97.6
	38	4	8.09	93.6
14.4	22	0	14.5	100
	39	4	13.5	93.4
24.0	23	0	23.0	95.8
	40	4	24.7	103
24.0 (Abiotic Control)	41	4	24.1	100

¹ Concentrations were corrected for change in test substance purity (90.49% to 86.9%) per Certificate of Analysis dated September 7, 2000.

² The limit of quantitation (LOQ) was 0.240 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00480 mg a.i./L) and the dilution factor of the matrix blank samples (50).

³ Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since fortified and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

**METHOD OUTLINE FOR THE ANALYSIS OF PFOS
IN FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX) SOLUTION**

Prepare matrix fortification samples by spiking the requisite volume of PFOS stock solutions directly into FETAX solution using gas-tight syringes and Class A volumetric flasks.



Dilute matrix fortification and test samples into the range of the calibration standards by partially filling Class A volumetric flasks with 50% methanol : 50% NANOpure[®] water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v). Add the appropriate volume of sample and bring the flask to volume with the dilution solvent. Process the matrix blank sample using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.



Ampulate samples and submit for LCMS analysis.

Figure 1. Analytical method flowchart for the analysis of PFOS in frog embryo teratogenesis assay-*Xenopus* (FETAX) solution.

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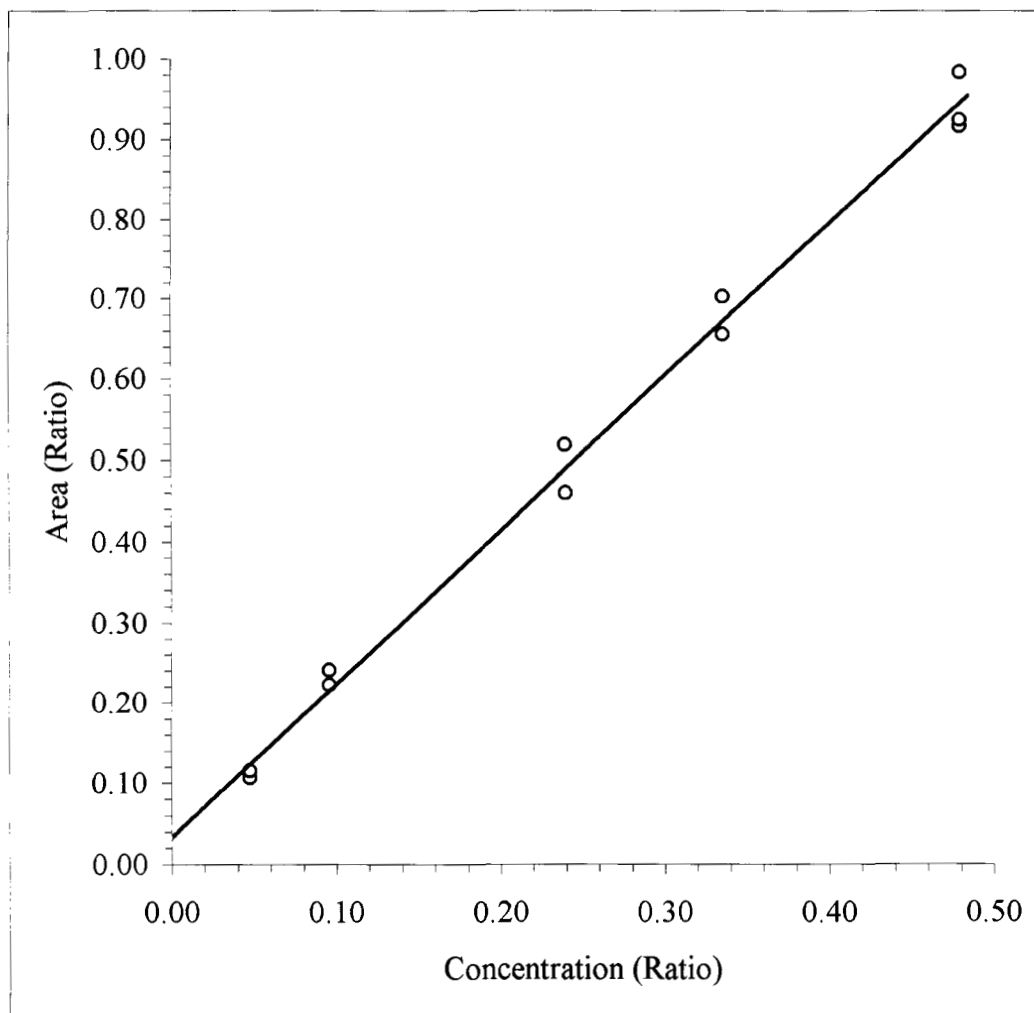


Figure 2. A typical calibration curve for PFOS. Slope = 1.9275; Intercept = 0.0260; $r = 0.9973$. Curve is weighted ($1/x$).

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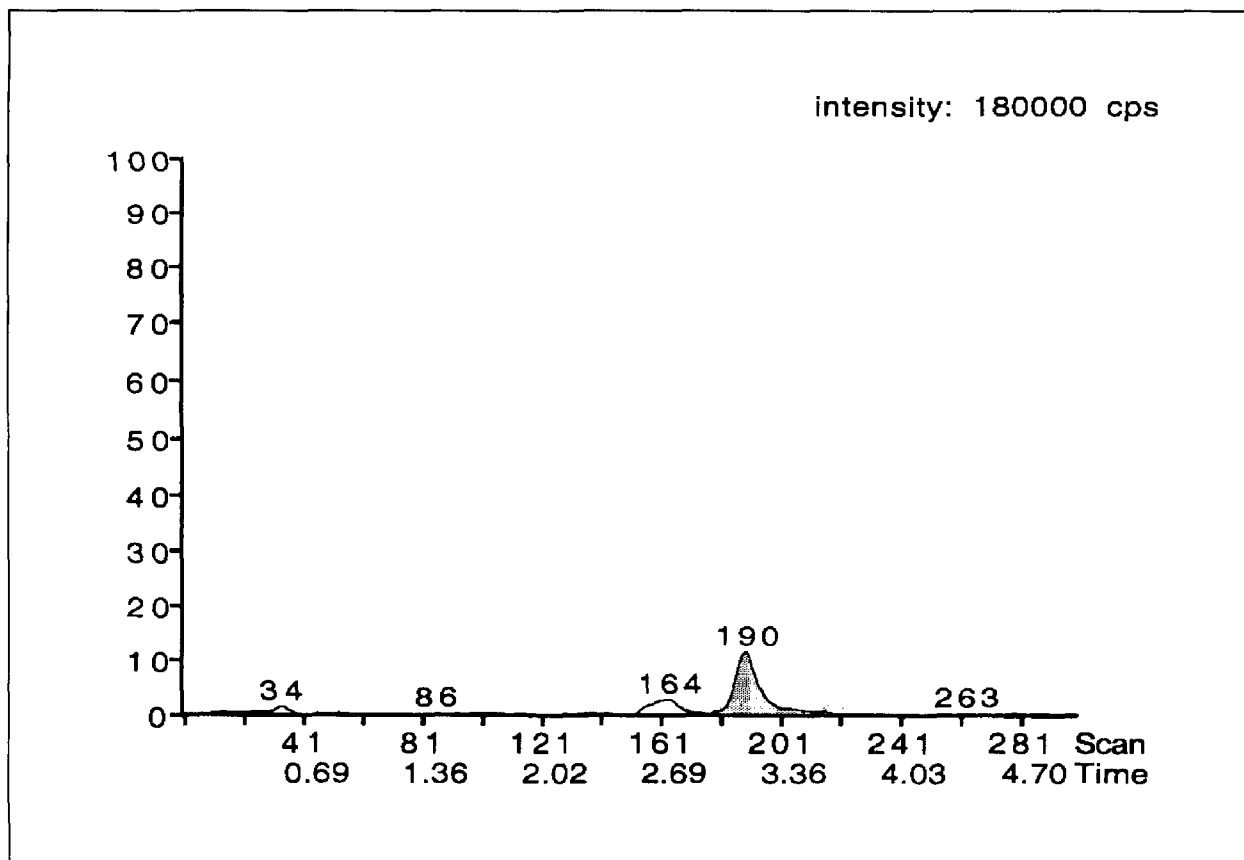


Figure 3. A representative ion chromatogram of a low-level (0.00480 mg a.i./L) PFOS standard.

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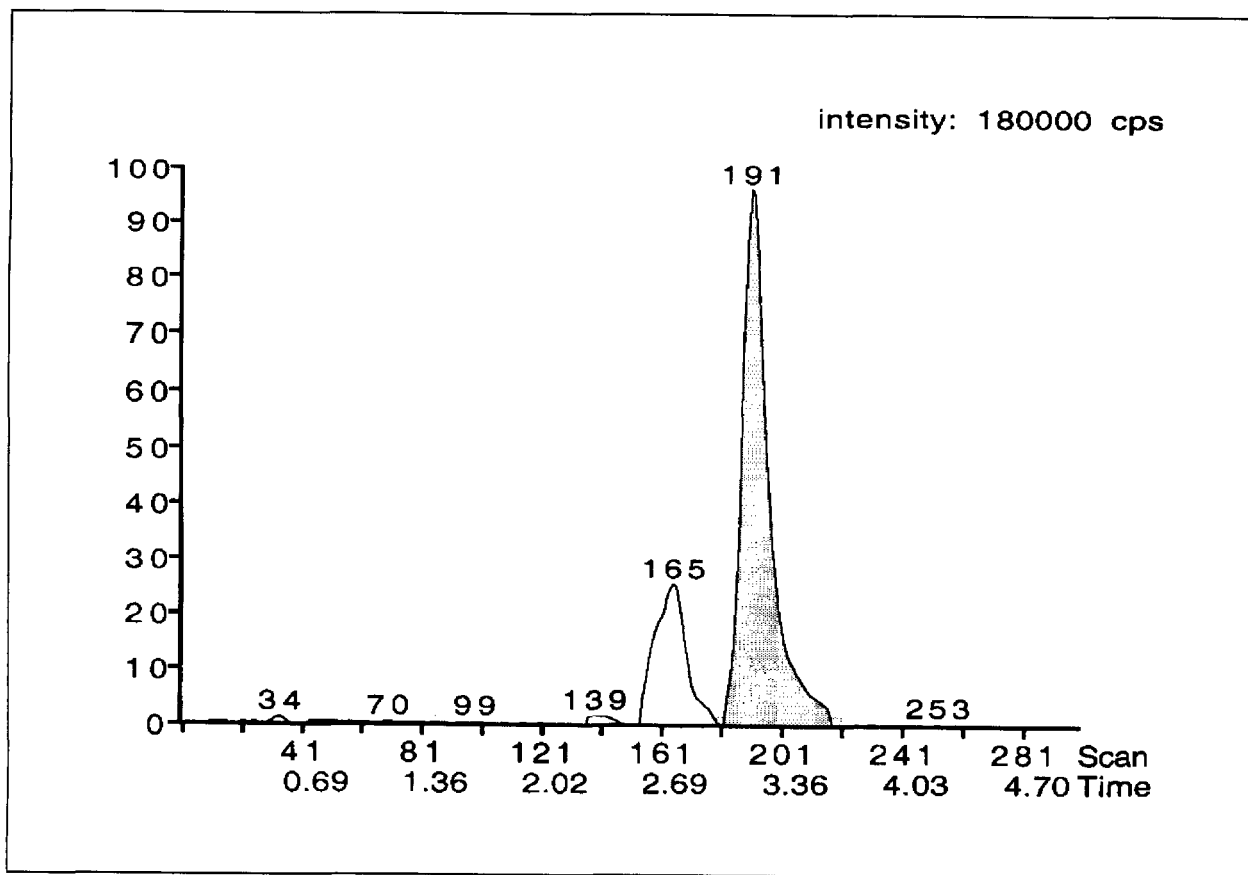


Figure 4. A representative ion chromatogram of a high-level (0.0480 mg a.i./L) PFOS standard.

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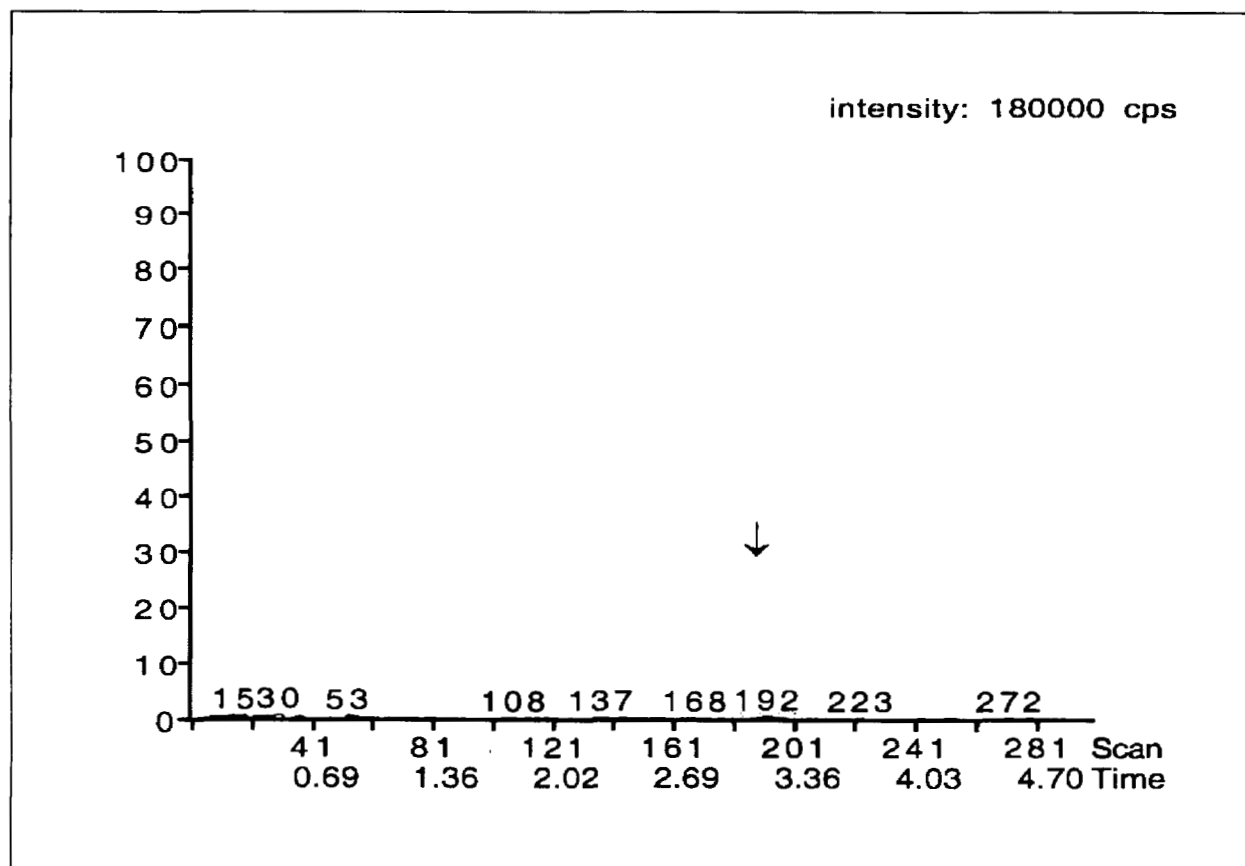


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-116-MAB-1). The arrow indicates the retention time of PFOS.

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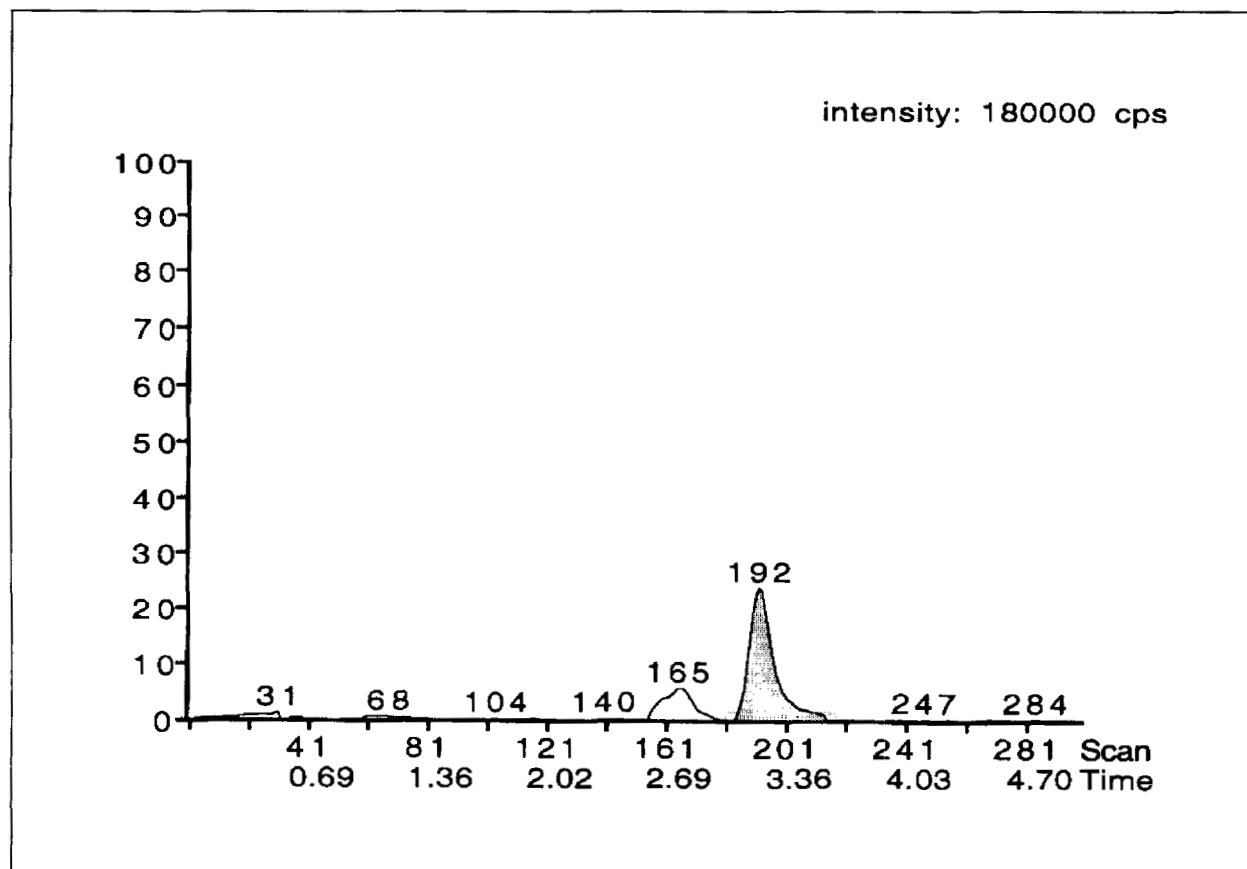


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-116-MAS-1, 0.480 mg a.i./L nominal concentration).

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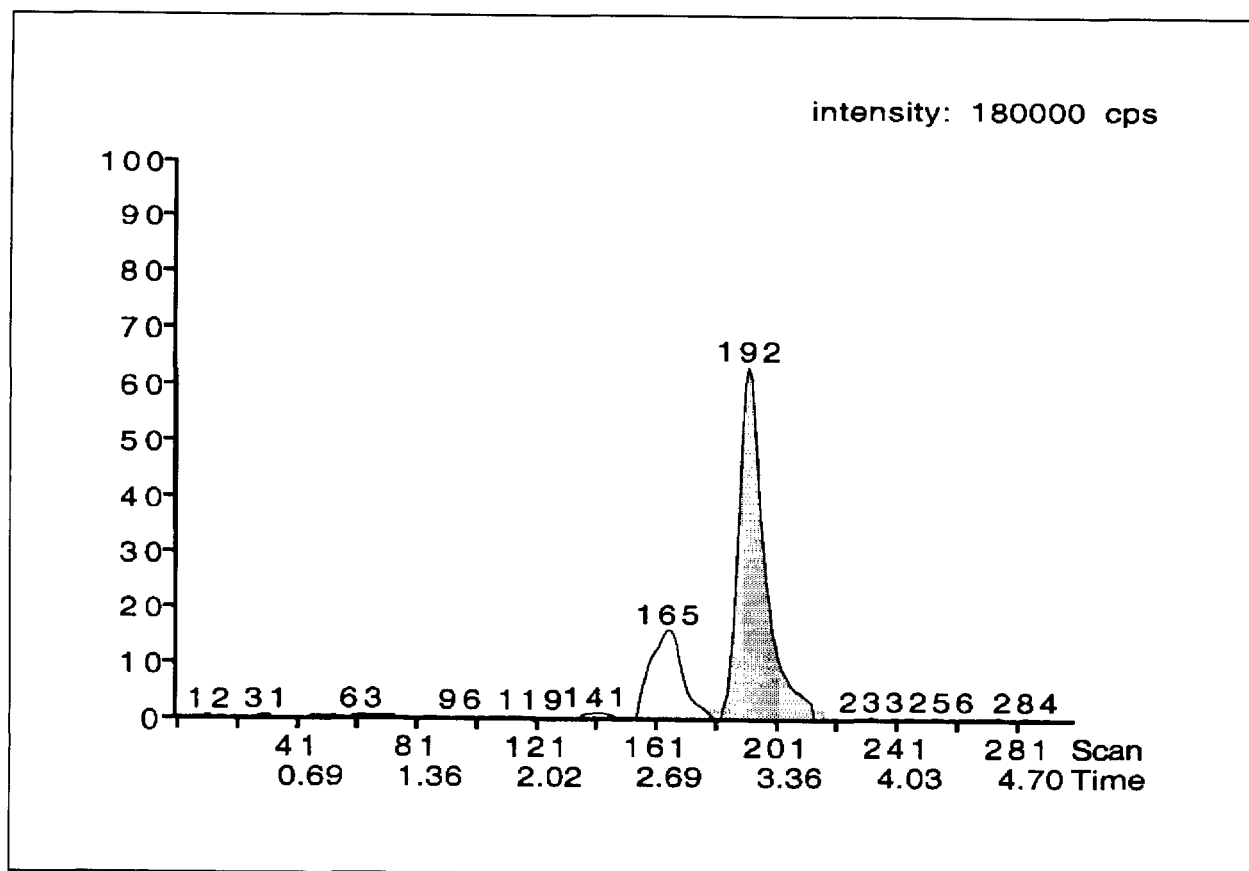


Figure 7. A representative ion chromatogram of a test sample (454A-116-7, 24.0 mg a.i./L nominal concentration).

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APPENDIX IV

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to change the test substance name at the Sponsor's request.
2. The nominal concentrations were recalculated since the Sponsor requested that the purity of the test substance be changed from 90.49% to 86.9%.

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APPENDIX V

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Director, Analytical Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Susan J. Palmer, Senior Biologist
5. Raymond L. VanHoven, Ph.D., Scientist