PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

ACUTE TOXICITY TO AQUATIC INVERTEBRATES (E.G.,DAPHNIA)

TEST SUBSTANCE

Identity: Perfluorooctanesulfonate; may also be referred to as PFOS or FC-95. (1-Octanesulfonic acid, 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-, potassium salt, CAS # 2795-39-3)

Remarks field: The test substance is a white powder. Sample was taken from 3M lot number 217. Sample was stored under ambient conditions prior to testing. Purity determined to be 90.49% by LC/MS, ^{1}H-HMR, ^{19}F-NMR and elemental analyses techniques.

METHOD

Method: OECD 202 and OPPTS 850.1010
Test type: Static acute
GLP: Yes
Year Completed: Study completed 1999. Report completed 2000
Species: *Daphnia magna*
Analytical monitoring: PFOS measured at 0, 24, 48-hours
Statistical methods: EC_{50} values calculated, when possible, by probit analysis, moving average method or binomial probability with non-linear interpolation using the computer software of C.E. Stephan.
Test daphnid source: Obtained from cultures maintained by Wildlife International Ltd., Easton, MD. Identification of the original brood stock was verified by the Academy of Natural Sciences, Philadelphia, PA.
Test daphnid age at study initiation: < 24-hours

Test conditions

Dilution water: 0.45 µm filtered well water
Dilution water chemistry (during the 4-week period immediately preceding the test):

hardness: 132 (128-136) mg/L as $CaCO_3$
alkalinity: 178 (176-178) mg/L as CaCO3
pH: 8.3 (8.2-8.3)
TOC: < 1.0 mg/L
Conductivity: 313 (310-315) µmhos/cm
Ca/Mg ratio: 35/13.5
Na/K ratio: 21.3/6.62

Lighting: Colortone® 50 fluorescent lights, intensity approximately 359 lux. Photoperiod of 16-hours light, 8-hours dark with a 30-minute transition period.

Stock and test solutions preparation: A primary stock solution was prepared in dilution water at 91 mg/L. It was mixed for ~19.5 hours prior to use. After mixing, the primary stock was proportionally diluted with dilution water to prepare the four additional test concentrations. All test solutions appeared clear and colorless.
Exposure vessels: 250 mL plastic beakers containing 240 mL of test solution. The approximate depth of test solution was 6.4 cm.
Number of replicates: two
Number of daphnids per replicate: ten
Number of concentrations: five plus a negative control
Water chemistry during the study:

Dissolved oxygen range (0 – 48 hours):
8.6 – 8.9 mg/L (control exposure)
8.6 – 9.1 mg/L (91 mg/L exposure)

pH range (0 – 48 hours)
8.2 – 8.5 (control exposure)
8.5 – 8.6 (91 mg/L exposure)

Test temperature range (0 – 48 hours)
19.5 – 20.2°C (control exposure)
19.3 – 20.1°C (91 mg/L exposure)

Element basis: mortality and immobilization
Method of calculating mean measured concentrations: arithmetic mean

RESULTS

Nominal concentrations: Bk control, 12, 20, 33, 55, 91 mg/L
Measured concentrations: <LOQ, 11, 20, 33, 56, 91 mg/L
Element value: 24-hour EC_{10} = 82 (81-83) mg/L
24-hour EC_{50} = >91 mg/L (C.I. not calculable)
24-hour EC_{90} = >91 mg/L (C.I. not calculable)
48-hour EC_{10} = 53 (<11->91) mg/L
48-hour EC_{50} = 61 (33-91) mg/L
48-hour EC_{90} = 63 (<11->91) mg/L
All element values based on mean measured concentrations

Statistical Evaluation: The EC_{50} values and 95% confidence intervals were calculated when possible by probit analysis, the moving average method or binomial probability with non-linear interpolation using the computer software of C.E. Stephan. The EC_{10} and EC_{90} values were calculated when possible using the Bruce-Versteeg method because there were less than two concentrations with partial mortality or immobility.

Analytical Methodology: Analyses of test solutions were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). When determining the concentration of the test substance in the test solutions, the same and most prominent peak response for perfluorooctanesulfonate was used. No attempt was made to quantify on the basis of individual isomeric components. The LOQ (limit of quantitation) was 4.58 mg/L in this study. The mean percent recovery of matrix fortifications analyzed concurrently during sample analysis was 96.2. Samples collected at test initiation had measured values from 85.5 to 112% of nominal. Measured values for samples taken at 24-hours ranged from 92.2 to 115% of nominal. Measured values for samples taken at 48-hours ranged from 91.6 to 106% of nominal.

Summary of analytical chemistry data:

Nominal Test Concentration, mg/L	Measured Duplicated Values at 0, 24, and 48-hours, Respectively, mg/L	Mean Measured Concentration, mg/L	Percent of Nominal
Negative Control	All < LOQ	<LOQ	-
12	10.5, 10.6, 11.5, 12.5, 10.9, 12.0	11	92
20	17.2, 18.1, 22.8, 21.6, 21.4, 18.8	20	100
33	30.2, 34.1, 34.0, 36.1, 31.3, 34.0	33	100
55	50.5, 49.9, 57.0, 63.0, 56.8, 56.4	56	102
91	87.6, 102, 90.1, 84.4, 88.7, 92.4	91	100

Biological observations after 48-hours: Daphnids in the negative control, the 11 and the 20 mg/L treatments appeared healthy and normal throughout the test with no mortality, immobility or overt clinical signs of toxicity. Five percent mortality was observed at 48-hours in the negative control. The effects noted in this study were mortality; no immobilization was noted at any test concentration.

Cumulative percent mortality:

Mean Measured Test Conc., mg/L	24-hours	48-hours
Neg. Control	0	5
11	0	0
20	0	0
33	0	0
56	0	35
91	35	100

Control response: satisfactory

CONCLUSIONS

The potassium perfluorooctanesulfonate 48-hour EC50 for *Daphnia magna* was determined to be 61 mg/L with a 95% confidence interval of 33-91 mg/L. The 48-hour noimmobilization and no observed effect concentration was 33 mg/L.

Submitter: 3M Company, Environmental Laboratory, P.O. Box 33331, St. Paul, Minnesota, 55133

DATA QUALITY

Reliability: Klimisch ranking 1

REFERENCES

This study was conducted at Wildlife International, Ltd. Easton, MD at the request of the 3M Company.

OTHER

Last changed: 5/3/00

PFOS:
A 48-HOUR STATIC ACUTE TOXICITY TEST
WITH THE CLADOCERAN (*Daphnia magna*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-104

3M LAB REQUEST NO. U2723

U. S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1010
and
OECD Guideline 202

AUTHORS:

Kurt R. Drottar
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: December 10, 1998

STUDY COMPLETION DATE: June 4, 1999

AMENDED REPORT DATE: April 26, 2000

Submitted to

3M Corporation
Environmental Laboratory
935 Bush Avenue
St. Paul, Minnesota 55144

Wildlife International Ltd.
8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-104

STUDY COMPLETION DATE: June 4, 1999

AMENDED REPORT DATE: April 26, 2000

This study was 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; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984, with the following exceptions:

The test substance was not characterized in accordance with full GLP compliance; 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 is being recharacterized in accordance with GLP.

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

STUDY DIRECTOR:

Kurt R. Drottar
Senior Biologist

4/26/00
DATE

SPONSOR APPROVAL:

Susan A. Beach
Sponsor

4/27/00
DATE

AMENDED

QUALITY ASSURANCE STATEMENT

This study was examined for 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; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. 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:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	February 15, 1999	February 15, 1999	February 16, 1999
Analytical Sample Preparation	February 16, 1999	February 17, 1999	February 19, 1999
Water Chemistry Measurements	February 17, 1999	February 17, 1999	February 19, 1999
Biological Data and Draft Report	March 17 and 18, 1999	March 18, 1999	March 29, 1999
Analytical Data and Draft Report	March 23 – 25, 1999	March 25, 1999	March 26, 1999
Final Report	June 4, 1999	June 4, 1999	June 4, 1999
Amended Report	April 20 and 25, 2000	April 25, 2000	April 25, 2000

James H. Coleman

James H. Coleman
Quality Assurance Representative

4-25-00

DATE

AMENDED

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-104

STUDY DIRECTOR:

Kurt R. Drottar
Senior Biologist

4/26/00
DATE

MANAGEMENT:

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

4/26/00
DATE

AMENDED

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPPRESENTATIVE:	Ms. Susan A. Beach
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-104
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 11, 20, 33, 56 and 91 mg a.i./L
TEST DATES:	Experimental Start – February 16, 1999 Biological Termination – February 18, 1999 Experimental Termination – February 18, 1999
LENGTH OF TEST:	48 Hours

TEST ORGANISM:	Neonate cladocerans (*Daphnia magna*)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. Cultures Easton, Maryland 21601
AGE OF TEST ORGANISMS:	< 24 hours at test initiation

48-HOUR EC50:	61 mg a.i./L
95% CONFIDENCE LIMITS:	33 and 91 mg a.i./L
NO MORTALITY/IMMOBILITY CONCENTRATION:	33 mg a.i./L
NO-OBSERVED-EFFECT-CONCENTRATION:	33 mg a.i./L

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INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from February 16, 1999 to February 18, 1999. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-104 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of PFOS to the cladoceran, *Daphnia magna*, during a 48-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Daphnids were exposed to a geometric series of five test concentrations and a negative (dilution water) control. Two replicate test chambers were maintained in each treatment and control group, with 10 daphnids in each test chamber for a total of 20 daphnids per test concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory rangefinding toxicity test. Nominal test concentrations selected were 12, 20, 33, 55 and 91 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, at approximately 24 hours, and at test termination.

Daphnids were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality/immobility and other clinical signs of toxicity were made at approximately 3, 24 and 48 hours after test initiation. Cumulative percent mortality and immobility observed in the treatment groups were used to estimate or calculate EC10, EC50 and EC90 values at 24 and 48 hours. The no mortality/immobility concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality, immobility and clinical observation data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*). The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines OPPTS Number 850.1010 (1), OECD Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (2) and ASTM Standard E-729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 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 March 9, 2000 indicated a purity of 90.49%. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 12, 20, 33, 55 and 91 mg a.i./L, based on a test substance purity of 90.49%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A primary stock solution was prepared in dilution water at a concentration of 91 mg a.i./L. The primary stock solution was mixed with an electric drum mixer with a stainless steel paddle for approximately 19.5 hours to aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with dilution water to prepare the four additional test concentrations. All test solutions appeared clear and colorless.

Test Organism

The cladoceran, *Daphnia magna*, was selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates and were selected for use in the test based upon past history of use and ease of culturing in the laboratory. Daphnid neonates used in the test were less than 24-hours old and were obtained from cultures maintained by Wildlife International Ltd., Easton, Maryland. Identification of the original brood stock was verified by the Academy of Natural Sciences, Philadelphia, PA.

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Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test. Adult daphnids in the cultures were held for at least 28 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. During the 14-day period preceding the test, water temperatures ranged from 19.9 to 20.4°C. The pH of the water ranged from 8.1 to 8.3, and dissolved oxygen ranged from 8.3 to 8.8 mg/L. Instrumentation used for water measurements are described in the *Environmental Conditions* section of this report.

Neonate daphnids were obtained for testing from individual adult daphnids which were observed to have no neonates present less than 24 hours prior to test initiation. The progeny from 10 adults were used in the test. At test initiation, the juvenile daphnids were collected from the cultures and indiscriminately transferred to 10-mL glass beakers. The daphnids then were transferred from the beakers to the test compartments. All transfers were performed below the air/water interface using a wide-bore pipet. Daphnids in the cultures were fed a mixture of yeast, Cerophyll®, and trout chow, as well as a suspension of the freshwater green alga, *Selenastrum capricornutum*. The adults were fed prior to test initiation, but neonates were not fed during the test.

Test Apparatus

Test chambers consisted of 250-mL plastic (Nalgene®) beakers containing 240 mL of test solution. The depth of test solution in a representative test chamber was 6.4 cm. Test chambers were indiscriminately placed in a temperature-controlled water bath set to maintain a temperature of 20 ± 1°C. Test chambers were labelled with the project number, test concentration and replicate.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity, and pH of the well water during the four-week period immediately preceding the test are presented in Appendix I.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water again was filtered (0.45 μm) to remove microorganisms and particles. The results of periodic analyses performed

to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix II.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 359 lux at the surface of the water. Light intensity was measured using a SPER Scientific Ltd. light meter.

Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was 20±1°C. The pH and dissolved oxygen content of the water were measured in each test chamber at test initiation, at approximately 24 hours after test initiation and at the end of the test. Hardness, alkalinity, specific conductance and total organic carbon (TOC) were measured in the dilution water at test initiation and at test termination.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4). Total organic carbon was measured using a Shimadzu Model 5000 TOC analyzer.

Observations

Observations were made to determine the numbers of mortalities and immobile organisms. The numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 3, 24, and 48 hours after test initiation.

Statistical Analyses

The 24 and 48-hour EC50 values and the 95% confidence intervals were calculated when possible by probit analysis, the moving average method or binomial probability with non-linear interpolation (5, 6, 7) using the computer software of C.E. Stephan (8). The no mortality/immobility concentration and NOEC were determined by inspection of the mortality, immobility and clinical observation data. In this study, EC10 and EC90 values were calculated when possible using the Bruce-Versteeg Method (9) because there were less than two concentrations with partial mortality or immobility (i.e., the probit method could not be used).

Analytical Chemistry

Samples were collected from mid-depth of each replicate test chamber at the beginning of the test, at approximately 24 hours and at test termination to measure concentrations of the test substance. The samples were collected in plastic (Nalgene®) bottles and analyzed immediately without storage. Analytical procedures used in the analysis of the samples are provided in Appendix III.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentration selected for use in this study were 12, 20, 33, 55 and 91 mg a.i./L. Samples collected at test initiation (0 Hours) had measured values that ranged from 85 to 112% of nominal. Samples collected at 24 hours had measured values that ranged from 92 to 113% of nominal, whereas measured values for samples taken at 48 hours ranged from 92 to 106% of nominal. When measured concentrations of the samples collected at test initiation, 24 hours, and at test termination were averaged, the mean measured concentrations for this study were 11, 20, 33, 56 and 91 mg a.i./L. Mean measured concentrations were used in the estimation or calculation of EC10, EC50 and EC90 values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures remained within the 20± 1 °C range established for the test. Dissolved oxygen concentrations remained ≥ 8.6 mg/L (95% of saturation) throughout the test. Measurements of pH ranged from 8.2 to 8.6 during the test.

Daily observations of mortality, immobility and other clinical signs observed during the test are shown in Table 3. Daphnids in the negative control appeared healthy and normal throughout the test, although 5% mortality was observed at 48 hours. Daphnids in the 11 and 20 mg a.i./L treatment groups also appeared normal with no mortality/immobility or overt clinical signs of toxicity. After 48 hours of exposure, mortality/immobility in the 33, 56 and 91 mg a.i./L treatment groups was 0, 35 and 100%, respectively. Although no mortality/immobility occurred in the 33 mg a.i./L treatment group, one daphnid was observed to be lethargic. Based on the 5% control mortality observed, this lethargy was not considered to be treatment-related. EC10, EC50 and EC90 values and 95% confidence limits at 24 and 48 hours were estimated or calculated from the mortality/immobility data, and are shown in Table 4.

CONCLUSIONS

The 48-hour EC50 value for *Daphnia magna* exposed to PFOS was 61 mg a.i./L with 95% confidence limits of 33 and 91 mg a.i./L. The no mortality/immobility concentration and NOEC were 33 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (draft), OPPTS Number 850.1010. *Aquatic invertebrate Acute Toxicity Test, Freshwater Daphnids.*

2 **Organisation for Economic Cooperation and Development.** 1984. *Daphnia sp. Acute Immobilization Test and Reproduction Test.* OECD Guideline for Testing of Chemicals. Guideline 202. Paris.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

5 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

8 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

9 **Bruce, R.D. and D.J. Versteeg.** 1992. A statistical procedure for modeling continuous toxicity data. Environmental Toxicology and Chemistry, Vol. II, pp 1485-1494.

Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Cladoceran, *Daphnia magna*
Dilution Water:	Well Water

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	A	0	<LOQ[1]	<LOQ	
	B	0	<LOQ		
	A	24	<LOQ		
	B	24	<LOQ		
	A	48	<LOQ		
	B	48	<LOQ		
12	A	0	10.5	11	92
	B	0	10.6		
	A	24	11.5		
	B	24	12.5		
	A	48	10.9		
	B	48	12.0		
20	A	0	17.2	20	100
	B	0	18.1		
	A	24	22.8		
	B	24	21.6		
	A	48	21.4		
	B	48	18.8		
33	A	0	30.2	33	100
	B	0	34.1		
	A	24	34.0		
	B	24	36.1		
	A	48	31.3		
	B	48	34.0		
55	A	0	50.5	56	102
	B	0	49.9		
	A	24	57.0		
	B	24	63.0		
	A	48	56.8		
	B	48	56.4		
91	A	0	87.6	91	100
	B	0	102		
	A	24	90.1		
	B	24	84.4		
	A	48	88.7		
	B	48	92.4		

[1] The limit of quantitation (LOQ) was 4.58 mg a.i./L.

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Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Sponsor	3M Corporation
Test Substance:	PFOS
Test Organism:	Cladoceran, *Daphnia magna*
Dilution Water:	Well Water

Mean Measured Concentration (mg a.i./L)	Replicate	0 Hour[1] Temp[2] (°C)	0 Hour[1] DO[3] (mg/L)	0 Hour[1] pH	24 Hours DO (mg/L)	24 Hours pH	48 Hours[4] Temp (°C)	48 Hours[4] DO (mg/L)	48 Hours[4] pH
Negative Control	A	19.6	8.9	8.2	8.6	8.4	20.2	8.6	8.5
	B	19.5	8.9	8.3	8.6	8.5	19.9	8.6	8.5
11	A	19.5	8.9	8.3	8.6	8.5	20.1	8.7	8.5
	B	19.4	8.9	8.3	8.6	8.5	20.1	8.6	8.5
20	A	19.4	8.9	8.3	8.6	8.5	20.1	8.6	8.5
	B	19.4	8.9	8.3	8.6	8.5	20.0	8.7	8.6
33	A	19.4	9.0	8.3	8.6	8.5	20.0	8.6	8.6
	B	19.4	9.0	8.3	8.6	8.5	20.1	8.6	8.6
56	A	19.3	9.1	8.4	8.6	8.5	20.0	8.7	8.6
	B	19.3	9.1	8.4	8.6	8.5	20.0	8.7	8.6
91	A	19.4	9.1	8.5	8.6	8.5	20.1	8.6	8.6
	B	19.3	9.1	8.5	8.6	8.5	20.0	8.7	8.6

[1] The 0-hour dilution water measurements for hardness, alkalinity, specific conductance and TOC were 136 mg/L as $CaCO_3$, 178 mg/L as $CaCO_3$, 315 μmhos/cm and < 1 mgC/L, respectively.

[2] Temperature measured continuously during the test ranged from approximately 19.5 to 20.5°C.

[3] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20°C in freshwater.

[4] The test termination dilution water measurements for hardness, alkalinity, specific conductance and TOC were 140 mg/L as $CaCO_3$, 180 mg/L as $CaCO_3$, 315 μmhos/cm and < 1 mgC/L, respectively.

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Table 3
Cumulative Percent Mortality/Immobility and Treatment-Related Effects[1]

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Cladoceran, *Daphnia magna*
Dilution Water: Well Water

Mean Measured Concentration (mg a.i./L)	Replicate	Daphnia/ Replicate	3 Hours			24 Hours			Percent Immobile and Dead	48 Hours			Percent Immobile and Dead
			Number Dead	Number Immobile	Effects	Number Dead	Number Immobile	Effects		Number Dead	Number Immobile	Effects	
Negative Control	A	10	0	0	10 AN	0	0	10 AN	0	1	0	9 AN	5
	B	10	0	0	10 AN	0	0	10 AN		0	0	10 AN	
11	A	10	0	0	10 AN	0	0	10 AN	0	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN		0	0	10 AN	
20	A	10	0	0	10 AN	0	0	10 AN	0	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN		0	0	10 AN	
33	A	10	0	0	10 AN	0	0	10 AN	0	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN		0	0	9AN,1C	
56	A	10	0	0	10 AN	0	0	9AN,1C	0	5	0	2AN,3C	35
	B	10	0	0	10 AN	0	0	10 AN		2	0	4AN,4C	
91	A	10	0	0	10 AN	3	0	5AN,2C	35	10	0	–	100
	B	10	0	0	10 AN	4	0	4AN,2C		10	0	–	

[1] Observed Effects: AN = Appears Normal, C = Lethargy.

AMENDED

Table 4

EC10, EC50 and EC90 Values

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Cladoceran, *Daphnia magna*
Dilution Water:	Well Water

Time	EC10 (mg a.i./L)	Lower 95% Confidence Limits	Upper 95% Confidence Limits	Statistical Method
24 Hours	82	81	83	Bruce-Versteeg
48 Hours	53	< 11	> 91	Bruce-Versteeg

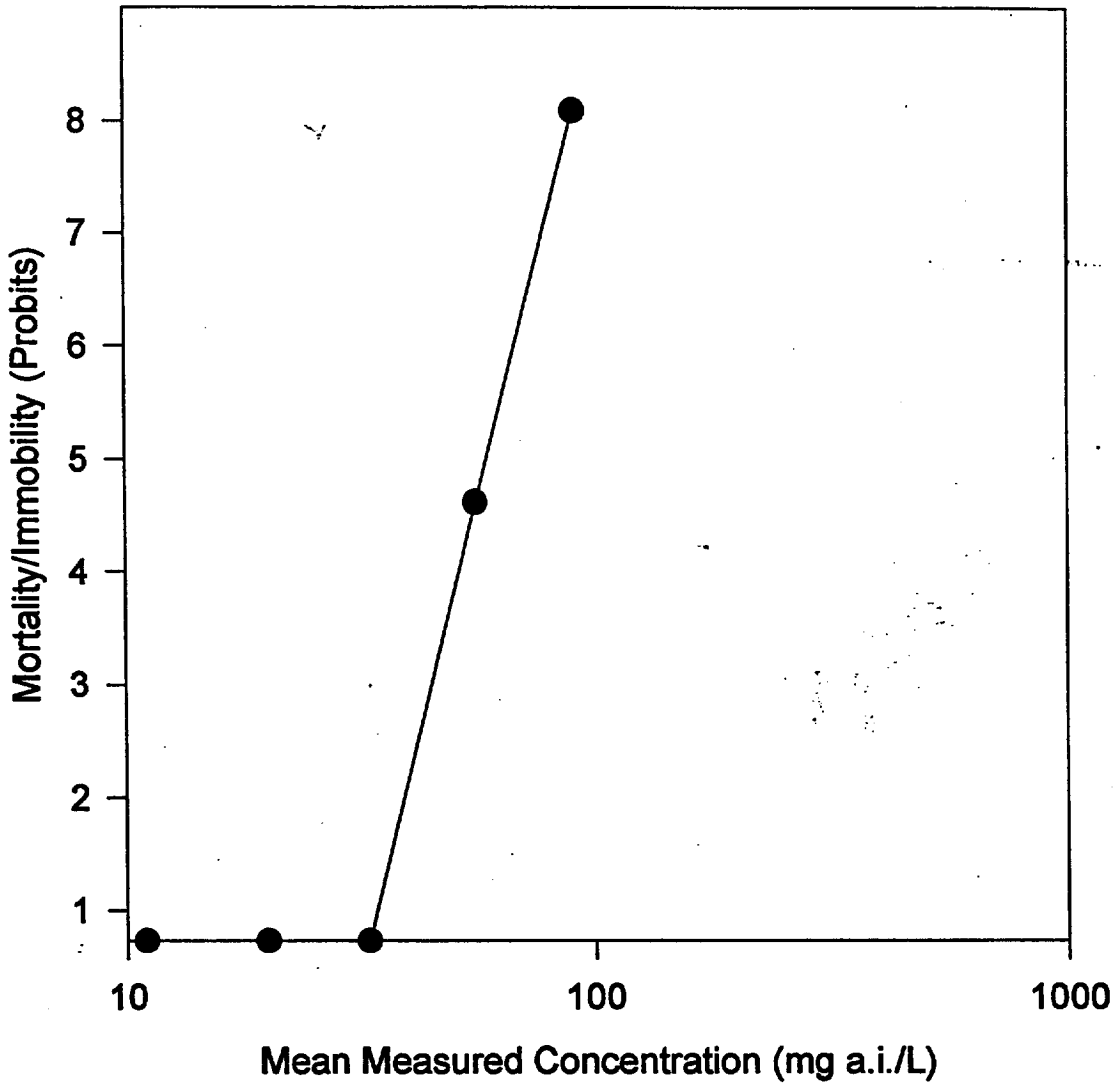
Time	EC50 (mg a.i./L)	Lower 95% Confidence Limits	Upper 95% Confidence Limits	Statistical Method
24 Hours	> 91	--[1]	--[1]	Visual Inspection
48 Hours	61	33	91	Binomial

Time	EC90 (mg a.i./L)	Lower 95% Confidence Limits	Upper 95% Confidence Limits	Statistical Method
24 Hours	> 91	--[1]	--[1]	Visual Inspection
48 Hours	63	< 11	> 91	Bruce-Versteeg

[1] Confidence limits could not be calculated with the mortality/immobility data obtained.

AMENDED

Figure 1. Concentration-Response Curve (48 Hour Data)



AMENDED

APPENDIX I

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Cladoceran, *Daphnia magna*
Dilution Water:	Well Water

	Mean	Range
Specific Conductance (μmhos/cm)	313 (N = 4)	310 – 315
Hardness (mg/L as $CaC0_3$)	132 (N = 4)	128 – 136
Alkalinity (mg/L as $CaC0_3$)	178 (N = 4)	176 – 178
pH	8.3 (N = 4)	8.2 – 8.3

APPENDIX II
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Well Water[1]

ANALYSIS	MEASURED CONCENTRATION		
Miscellaneous Measurements			
Total Dissolved Solids		286	mg/L
Ammonia Nitrogen	<	0.050	mg/L
Total Organic Carbon[2]	<	1.0	mg/L
Total Cyanide	<	10.0	µg/L
Organochlorines and PCBs			
Aldrin	<	0.005	µg/L
Alpha BHC	<	0.005	µg/L
Beta BHC	<	0.005	µg/L
Delta BHC	<	0.005	µg/L
Gamma BHC (Lindane)	<	0.006	µg/L
Chlordane	<	0.025	µg/L
DDD, pp'	<	0.006	µg/L
DDE, pp'	<	0.005	µg/L
DDT, pp'	<	0.008	µg/L
Dieldrin	<	0.005	µg/L
Endosulfan, A	<	0.005	µg/L
Endosulfan, B	<	0.005	µg/L
Endosulfan Sulfate	<	0.018	µg/L
Endrin	<	0.010	µg/L
Endrin Aldehyde	<	0.005	µg/L
Heptachlor	<	0.005	µg/L
Methoxychlor	<	0.007	µg/L
Heptachlor Epoxide	<	0.005	µg/L
Toxaphene	<	0.500	µg/L
PCB-1016	<	0.260	µg/L
PCB-1221	<	0.260	µg/L
PCB-1232	<	0.260	µg/L
PCB-1242	<	0.720	µg/L
PCB-1248	<	0.720	µg/L
PCB-1254	<	0.720	µg/L
PCB-1260	<	0.720	µg/L
Metals and Other Inorganics			
Aluminum[3]	<	100	µg/L
Arsenic[3]	<	25.0	µg/L
Beryllium[3]	<	0.50	µg/L
Cadmium[3]	<	1.0	µg/L
Calcium[3]		35.0	mg/L
Chromium[3]	<	2.0	µg/L
Cobalt[3]	<	1.0	µg/L
Copper[3]	<	20.0	µg/L
Iron[3]	<	100	µg/L
Lead[3]	<	10.0	µg/L
Magnesium[3]		13.5	mg/L
Manganese[3]	<	1.0	µg/L
Mercury	<	0.20	µg/L
Molybdenum[3]	<	2.0	µg/L
Nickel[3]	<	2.0	µg/L
Potassium[3]		6.62	mg/L
Selenium[3]	<	25.0	µg/L
Silver[3]	<	1.0	µg/L
Sodium[3]		21.3	mg/L
Zinc[3]	<	20.0	µg/L

1 Analyses performed by QST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.
2 Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.
3 Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

APPENDIX III

THE ANALYSIS OF PFOS IN FRESHWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-104

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 48-Hour Static Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-104

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

1-25-00
DATE

MANAGEMENT:

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

4/26/00
DATE

AMENDED

Introduction

Freshwater samples were collected from a static acute aquatic toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the cladoceran (*Daphnia magna*). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-104. The analyses of these water samples were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on February 16, 17 and 18, 1999 and were analyzed on each sample receipt day.

Test Substance and Internal Standard

The test substance used for the analytical portion of 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 was stored under ambient conditions.

Analytical Method

The method used for the analysis of the freshwater samples was 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 the 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 100LC Mass Spectrometer equipped with a Perkin-

Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (100 mm x 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 0.00915 to 0.0457 mg a.i./L were analyzed with the samples. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentrations 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 4.58 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (500).

Matrix Blank and Fortification Samples

Three 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 chromatogram of a matrix blank is presented in Figure 5.

Freshwater was fortified at 9.15, 45.7 and 91.5 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 96.2%. A representative chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-104-27, nominal concentration of 12 mg a.i./L in freshwater.

Initial Volume: 0.100 mL

Final Volume: 50.0 mL

Dilution Factor: 500

PFOS Peak Area: 302788

Internal Standard Peak Area: 585991

Peak Area Ratio: 0.5167

Calibration curve equation.

Slope: 0.02117

Intercept: 0.05525

Curve is linear.

$$\text{PFOS (µg a.i./L) at instrument} = \frac{\text{Peak area ratio} - \text{(Y-intercept)}}{\text{Slope}}$$

$$= \frac{0.5167 - 0.05525}{0.02117}$$

$$= 21.8$$

$$\text{PFOS (mg a.i./L) in sample} = \frac{\text{PFOS (µg a.i./L) at instrument} \times \text{Dilution Factor}}{1000}$$

$$= \frac{21.8 \times 500}{1000}$$

$$= 10.9$$

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$$\text{Percent of Nominal Concentration} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

Calculated recovery: 91.6%

Note: manual calculation may differ.

RESULTS

Sample Analysis

Freshwater samples were collected from the acute toxicity study with the cladoceran (*Daphnia magna*) at test initiation, February 16, 1999 (Hour 0), on February 17, 1999 (Hour 24), and at test termination, February 18, 1999 (Hour 48). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 85.5 to 112% of the nominal concentrations. Samples collected at Hour 24 had a measured concentration range of 92.2 to 115% of nominal values. Samples collected at test termination (Hour 48) had a measured concentration range of 91.6 to 106% of nominal values (Table 4). A representative chromatogram of a test sample is shown in Figure 7.

Table 1

Typical LC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C_{18} column (100 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	10.0 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	50.0 µL
PFOS RETENTION TIME:	Approximately 7.1 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 4.9 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of PFOS[1]
Number (454A-104-)	Type	(mg a.i./L)
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ

[1] The limit of quantitation (LOQ) was 4.58 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (500).

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Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-104-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered
	Fortified	Measured	
MAS-1	9.15	8.42	92.0
MAS-4	9.15	9.72[1]	106
MAS-7	9.15	9.15	100
MAS-2	45.7	40.3	88.1
MAS-5	45.7	48.1	105
MAS-8	45.7	45.1	98.7
MAS-3	91.5	78.7	86.0
MAS-6	91.5	84.0	91.8
MAS-9	91.5	89.7	98.0
		Mean	= 96.2
		Standard Deviation	= 7.11
		CV	= 7.39
		N	= 9

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

[1] Mean result of duplicate injections of the rediluted original sample. Original result (5.48 mg a.i./L) was not included in the statistical analysis of the data.
Duplicate results for 454A-104-MAS-4 are 9.24 and 10.2.

AMENDED

Table 4

Measured Concentrations of PFOS in Freshwater Samples from a Cladoceran Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-104-)	Sampling Time (Hours)	PFOS Measured Concentration[1] (mg a.i./L)	Percent of Nominal
0.0	1	0	< LOQ	--
	2	0	< LOQ	--
	13	24	< LOQ	--
	14	24	< LOQ	--
	25	48	< LOQ	--
	26	48	< LOQ	--
12	3	0	10.5	88.5
	4	0	10.6	89.5
	15	24	11.5	96.8
	16	24	12.5	105
	27	48	10.9	91.6
	28	48	12.0	101
20	5	0	17.2	85.5
	6	0	18.1	90.2
	17	24	22.8	113
	18	24	21.6	107
	29	48	21.4	106
	30	48	18.8	93.6
33	7	0	30.2	91.7
	8	0	34.1	104
	19	24	34.0	103
	20	24	36.1	110
	31	48	31.3	95.1
	32	48	34.0	103

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

[1]The limit of quantitation (LOQ) was 4.58 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (500).

[2]Mean result of duplicate injections of the rediluted original sample. Original result (113 mg a.i./L) was not included in the statistical analysis of the data.

Duplicate results for 454A-104-23 are 90.6 and 89.6.

Table 4 (Continued)

Measured Concentrations of PFOS in Freshwater Samples from a Cladoceran Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-104-)	Sampling Time (Hours)	PFOS Measured Concentration[1] (mg a.i./L)	Percent of Nominal
55	9	0	50.5	92.1
	10	0	49.9	90.9
	21	24	57.0	104
	22	24	63.0	115
	33	48	56.8	104
	34	48	56.4	103
91	11	0	87.6	95.7
	12	0	102	112
	23	24	90.1[2]	98.5
	24	24	84.4	92.2
	35	48	88.7	96.9
	36	48	92.4	101

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

[1]The limit of quantitation (LOQ) was 4.58 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (500).

[2]Mean result of duplicate injections of the rediluted original sample. Original result (113 mg a.i./L) was not included in the statistical analysis of the data.
Duplicate results for 454A-104-23 are 90.6 and 89.6.

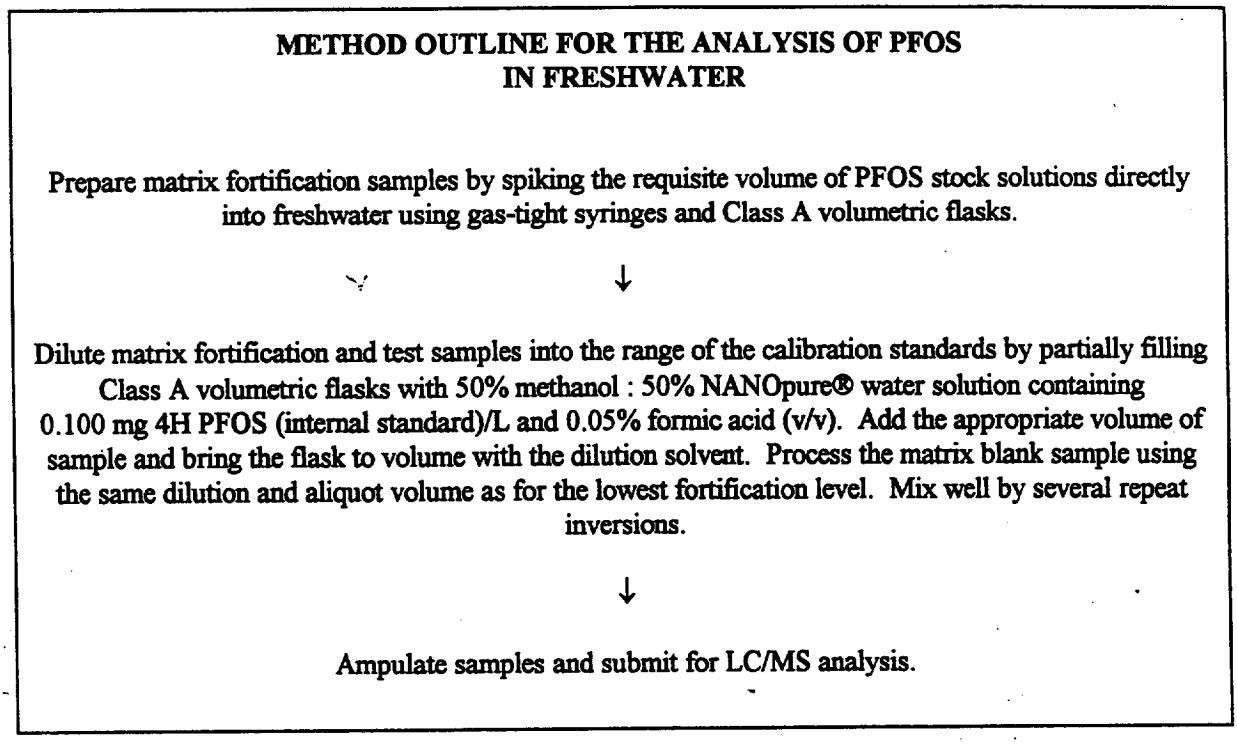


Figure 1. Analytical method flowchart for the analysis of PFOS in freshwater.

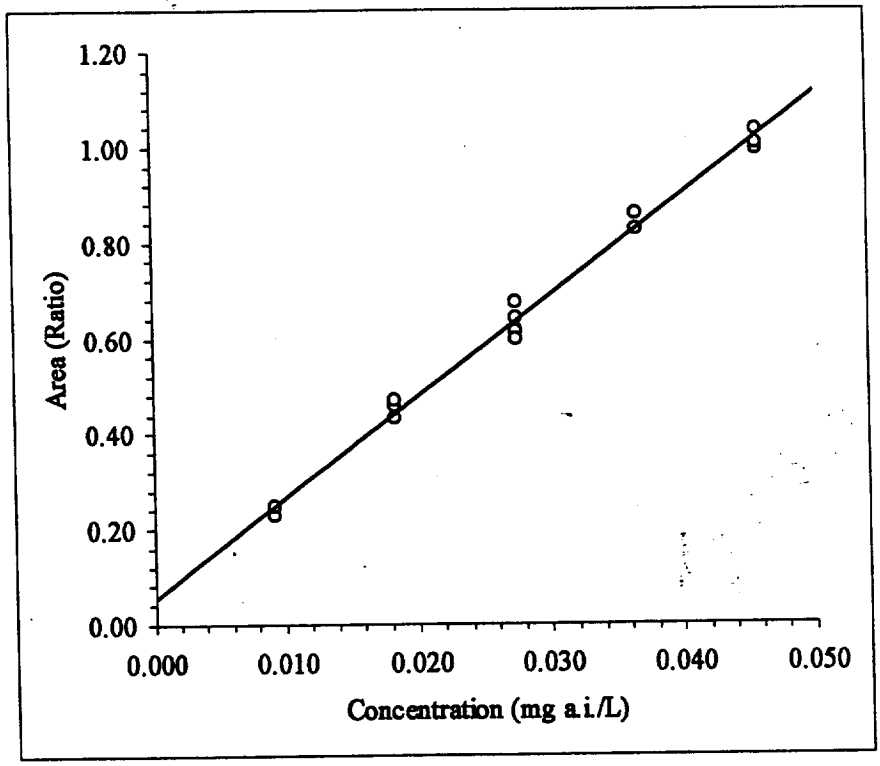


Figure 2. A typical calibration curve for PFOS. Slope = 0.02117; Intercept = 0.05525; r = 0.9961.

AMENDED

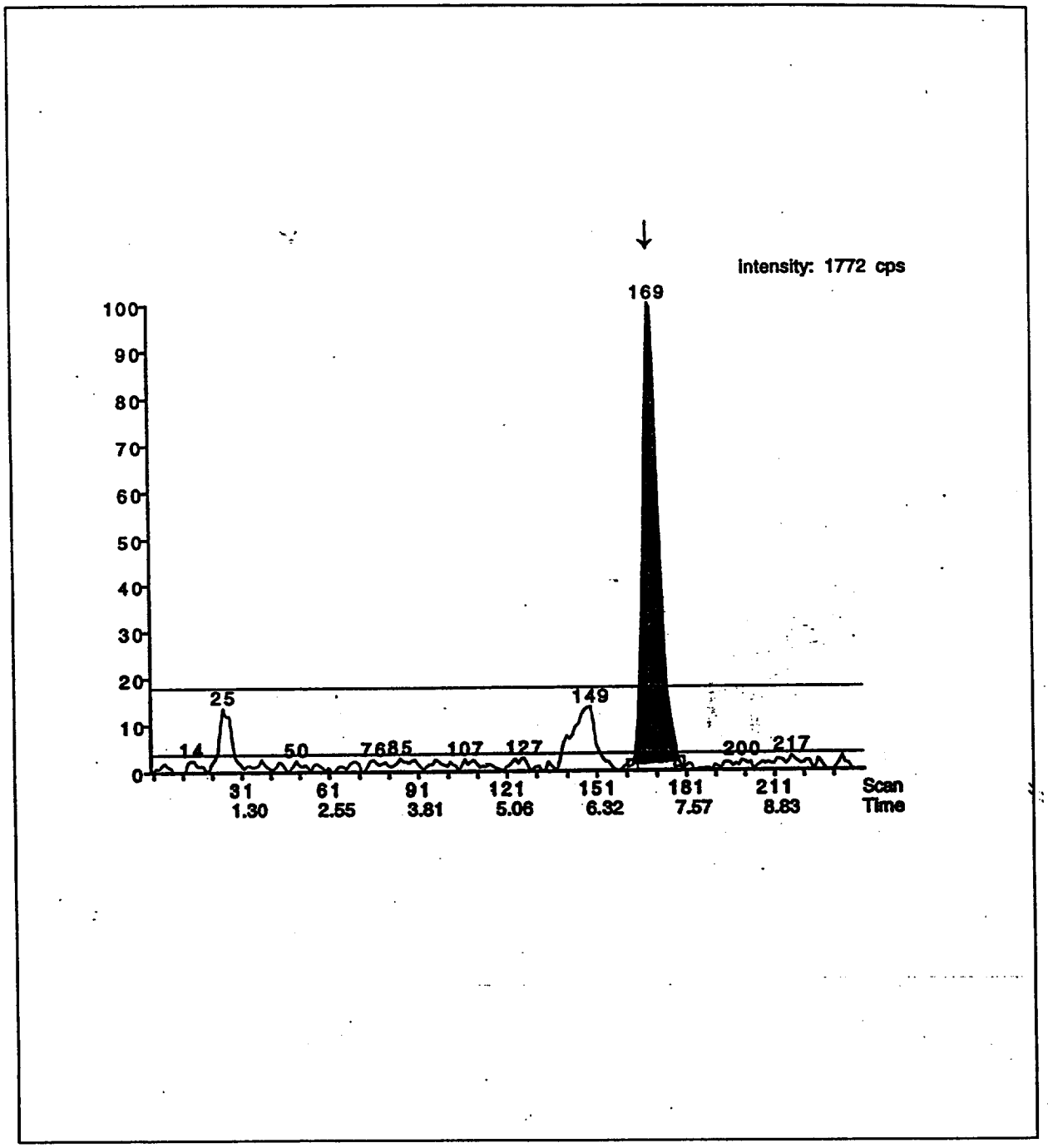


Figure 5. A representative chromatogram of a matrix blank sample (454A-104-MAB-3). The arrow indicates the retention time of PFOS.

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 add the proposed experimental start and termination dates, test concentrations and test substance number.
2. The protocol was amended to delete annual feed analyses.
3. The protocol was amended to add the analytical method.
4. The protocol was amended to change the statistical analyses.

APPENDIX VI

Report Amendment

1. Original Report: Pages 1-4, 6 and 23

   Amendment: The pages were changed to include the amended report date, revised page numbers, and new signatures and dates due to the addition of the report amendment as Appendix VI.

   Reason: To reflect the issuing of an amended report.

2. Original Report: Page 2

   Amendment: The compliance statement was revised.

   Reason: To clarify how the test substance was characterized.

3. Original Report: Page 9

   Amendment: Information provided by the Sponsor reflecting the reanalysis of the test substance, including the reanalysis date and the purity, was added to the Test Substance section.

   Reason: To reflect the current test substance information provided by the Sponsor.

4. Original Report: Entire report

   Amendment: All test substance concentrations were changed to reflect the purity of the test substance as determined by the Sponsor in a reanalysis of the test substance (FC-95, Lot 217). Test concentrations originally were based on the reported purity of 98.9%. The certificate of analysis dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations, including nominal concentrations, measured concentrations, and EC values, were recalculated and reported as mg a.i./L based on the 90.49% purity.

   Reason: To report the results of the test based on the test substance purity of 90.49% at the request of the Sponsor.

AMENDED

APPENDIX VI

-Continued-

Report Amendment

AMENDMENT SIGNATURES:

Kurt R. Drottar
Study Director

4/26/00
DATE

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

4/26/00
DATE

REVIEWED BY:

James H. Coleman
James H. Coleman
Quality Assurance

4-26-00
DATE

AMENDED

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

PROTOCOL NO.: 454/120198/DAP-48H1/SUB454 **AMENDMENT NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454A-104

EFFECTIVE DATE: December 21, 1998

AMENDMENT: Page 2

Add: Experimental Start Date: 2/16/99
Experimental Termination Date: 2/18/99
Test Concentrations: Negative Control, 13, 22, 36, 60 and 100 mg a.i./L
Test Substance No.: 4675

REASON: The above information was not known when the protocol was signed by the Study Director.

AMENDMENT: Test Organism, Page 5

Delete: Feed (YCT) provided to daphnids in the cultures will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study.

REASON: Historical analyses of Wildlife International Ltd. aquatic feed have shown that no contaminants are present at levels known to be capable of interfering with the study.

AMENDMENT: APPENDIX II, Page 14

Add: Liquid Chromatography Mass Spectrometry (LCMS) Method for the Determination of Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) in Freshwater, Filtered Saltwater and Algal Medium.

REASON: To add the analytical method to be used in the study.

QA Reviewed 2/10/99 LRM

STUDY DIRECTOR — DATE 2/10/99

LABORATORY MANAGEMENT — DATE 2/10/99

SPONSOR'S REPRESENTATIVE — DATE 2/16/99

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

PROTOCOL NO.: 454/120198/DAP-48H1/SUB454 **AMENDMENT NO.:** 2

SPONSOR: 3M Corporation **PROJECT NO.:** 454A-104

EFFECTIVE DATE: March 9, 1999

AMENDMENT: Data Analysis, Page 8

Change: When the dose-response pattern allows calculation of EC10, EC50 and EC90 values, the data will be analyzed using the computer software of C.E. Stephan (6).

To: When the dose-response pattern allows calculation of EC10, EC50 and EC90 values, the data will be analyzed using the computer software of C.E. Stephan (6), or another appropriate methodology.

REASON: The computer software of C.E. Stephan does not calculate EC10 or EC90 values.

STUDY DIRECTOR	DATE 3/11/99
LABOARATORY MANAGEMENT	DATE 3/11/99
SPONSOR'S REPRESENTATIVE	DATE 4/1/99

QA Reviewed 3/10/99 MM

PROTOCOL

PFOS: A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1010

and

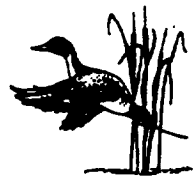
OECD Guideline 202

3M Lab Request No. U2723

Submitted to

3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133

WILDLIFE INTERNATIONAL LTD.



8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

December 1, 1998

PROTOCOL NO.: 454/120198/DAP-48H1/SUB454 3M LAB REQUEST NO. U2723

PFOS: A 48-HOUR STATIC ACUTE TOXICITY TEST
WITH THE CLADOCERAN (*Daphnia magna*)

SPONSOR:	3M Corporation Environmental Laboratory P.O. Box 33331 St. Paul, Minnesota 55133
SPONSOR'S REPRESENTATIVE:	Ms. Susan A. Beach
TESTING FACILITY:	Wildlife International Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Kurt Drottar Senior Aquatic Biologist
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Director of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: _____ Experimental Termination Date: _____

Project No.: 454A-104

Test Concentrations: _____

Test Substance No.: _____ Reference Substance No. (if applicable): _____

PROTOCOL APPROVAL

STUDY DIRECTOR	DATE 12/10/98
LABORATORY MANAGEMENT	DATE 12/10/98
SPONSOR'S REPRESENTATIVE	DATE 12/7/98

INTRODUCTION

Wildlife International Ltd. will conduct a static acute toxicity test with the cladoceran, *Daphnia magna*, for the Sponsor at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1010 (1), in OECD Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (2) and ASTM Standard E-729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVES

The objective of this study is to determine the acute effects of a test substance on the cladoceran, *Daphnia magna*, under static test conditions for a period of 48 hours.

EXPERIMENTAL DESIGN

Daphnids will be exposed to a geometric series of at least five test concentrations and a negative (dilution water) control for 48 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 neonate daphnids in each chamber so that a total of 20 neonate daphnids are exposed in each treatment and control group.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target concentrations need not exceed 1000 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each concentration of test substance used in the definitive test will be at least 60% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Water samples from each test chamber will be collected at specified intervals for analysis of the test substance. Results of the analyses will be used to calculate mean measured test concentrations.

To control bias, neonate daphnids will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. EC10, EC50 and EC90 values will be calculated, when possible, based on the number of dead or immobilized daphnids observed in each test concentration after each 24-hour interval of exposure. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP). The Sponsor is responsible for providing Wildlife International Ltd. written verification that the test substance has been characterized according to GLPs prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International Ltd., it will be noted in the compliance statement of the final report. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix I) is to be used to provide information necessary for GLP compliance.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism directly into dilution water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

Test Organism

The cladoceran, *Daphnia magna*, has been selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Daphnid neonates to be used in the test will be less than 24 hours old and will be obtained from cultures maintained at Wildlife International Ltd., Easton, Maryland. The identity of the species will be verified by the supplier of the

original culture or by Wildlife International Ltd. personnel using appropriate taxonomic keys such as Pennak (4).

Daphnids will be cultured in water from the same source and at approximately the same temperature as will be used during the test. Daphnids in the cultures producing neonates for the test will be held for at least 10 days prior to collection of the neonates for testing. Adult daphnids in the culture will produce an average of at least 3 young per adult per day over the 7 day period prior to the test. Neonates from daphnids that show signs of disease or stress will not be used as test organisms. Daphnids in holding that produce ephippia also will not be used to supply neonates for testing.

Daphnids in the cultures will be fed once daily. The diet will be a mixture of yeast, Cerophyll®, and trout chow (YCT), supplemented with a suspension of the freshwater green alga *Selenastrum capricornutum*. Adults are fed during the 24-hour period prior to test initiation, but neonates will not be fed during the test. Feed (YCT) provided to daphnids in the cultures will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study. Specifications for acceptable levels of contaminants in daphnid diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Neonates will be obtained for testing from at least three individual adults. Prior to test initiation, the neonates will be collected from cultures and transferred to small containers. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette.

Dilution Water

The water used for culturing and testing will be obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 μm in order to remove fine particles. Water used for culturing and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, μmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the water and results of the analyses will be summarized in the final report.

Test Apparatus

Test chambers will consist of polyethylene bottles. The size of bottle and the volume of test solution will be sufficient to meet the requirements of sampling for analytical verification of test substance concentrations and routine water chemistry analyses. Test chambers will be indiscriminately positioned in a temperature-controlled water bath or an environmental chamber. Test chambers will be labelled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation and at test termination with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be 20 ± 1 °C. Temperature will be measured in each test chamber at the beginning and end of the test using a hand-held thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a hand-held thermometer prior to test initiation.

Dissolved oxygen will be measured in all replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in all replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Fisher Accumet Model 915 pH meter, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken at the next sampling interval, then discontinued.

Hardness, pH, alkalinity, specific conductance and total organic carbon (TOC) will be measured in the dilution water at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Total organic carbon will be measured on a Shimadzu Model 5000 TOC analyzer, or equivalent.

Biological Observations

Observations of mortality, immobilization and clinical signs of toxicity will be made between 0-24 hours, and at 24 and 48 hours ± 1 hour. Daphnids not able to swim within 15 seconds after gentle agitation of the test chamber will be considered immobile.

Sampling for Analytical Measurements

Water samples will be collected from each test chamber at the beginning of the test and at 24 and 48 hours (± 1 hour) to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately, or placed in an appropriate storage container (e.g., polyethylene or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0 Hours	24 Hours	48 Hours
Control	2	2	2
Solvent Control (if needed)	2	2	2
Level 1-Low Concentration	2	2	2
Level 2	2	2	2
Level 3	2	2	2
Level 4	2	2	2
Level 5-High Concentration	2	2	2
Totals	14	14	14

Total Number of Verification Samples = 42

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon methodology provided by the Sponsor and identified in Appendix II. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of EC10, EC50 and EC90 values, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the EC values and the 95% confidence intervals by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7, 8 and 9). The EC values will be calculated using mortality/immobility data collected at 24 and 48 hours. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. History of the test organism, culture and holding records.
5. Stock solution calculation and preparation.
6. Daily observations.
7. Water chemistry results (e.g., hardness and alkalinity).
8. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.
9. Statistical calculations.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International Ltd. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.

7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, results of latest feed analyses, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration response curve for each period an EC50 is calculated, when sufficient data exists. If the data is conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.
14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (OCDE/GD (92) 32, Environment Monograph No. 45); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site or at an alternative location to be specified in the final report.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1010: *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids.*

2 **Organisation for Economic Cooperation and Development.** 1984. *Daphnia sp. Acute Immobilization Test and Reproduction Test.* OECD Guideline for Testing of Chemicals. Guideline 202. Paris.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **Pennak, R.W.** 1978. Freshwater Invertebrates of the United States. 2nd Ed. 365 p.

5 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

7 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

8 **Thompson, W.R.** 1974. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

9 **Stephan, C.E.** 1977. Methods for Calculating an LC50, *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634. Pp 65-84.

APPENDIX I
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): PFOS (Perfluorooctane Sulfonic Acid Potassium Salt

Reference Standard (if applicable): Analytical Standard: N/A

Internal Standard: 1,1,2,2H,H,H,H Perfluorooctane Sulfonic Acid

Test Substance Sample Code or Batch Number: Lot 217

Test Substance Purity (% Active Ingredient): 98.9 Expiration Date: 2008

II. Test Substance Characterization

Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? Yes ____ No X

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient room temperature

Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? Yes ____ No X

Other pertinent stability information: _____

IV. Test Concentrations:

X Adjust test concentration to 100% a.i. based upon the purity (%) given above.

____ Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:

Mammalian: Rat LD50 251 mg/kg Mouse LD50 N/A

Aquatic:	Invertebrate Toxicity (EC/LC50)	Fish Toxicity (LC50)
	Daphnia magna: 27 mg/L	Rainbow Trout: 11 mg/L
	Daphnia magna: 50 mg/L	Fathead Minnow: 38 mg/L

Other Toxicity Information (including findings of chronic and subchronic tests):

Please see MSDS

APPENDIX II

Analytical Method to be Provided by Sponsor