PFOS: A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH *Lumbriculus variegatus*

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-252A
3M ENVIRONMENTAL LABORATORY PROJECT NUMBER: E07-0084

ASTM Standard E729-96

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STUDY INITIATION DATE: February 1, 2007

STUDY COMPLETION DATE: March 15, 2007

SUBMITTED TO:

3M Corporation
Environmental Laboratory
3M Center
Building 0260-05-N-17
Maplewood, MN 55144

Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601 USA
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Lumbriculus variegatus*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-252A

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STUDY COMPLETION: March 15, 2007

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and 792, 17 August 1989) with the following exceptions:

Periodic analyses of well water for potential contaminants were performed using a certified laboratory and standard U.S. EPA analytical methods.

STUDY DIRECTOR:

Tui Minderhout

Tui Minderhout, Ph.D
Senior Biologist

March 15, 2007
Date

SPONSOR APPROVAL:

Sponsor Representative

3/20/2007
Date

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:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Protocol	January 22, 2007	January 22, 2007	February 12, 2007
Initial Trial 454A-252			
Test Substance Preparation	February 2, 2007	February 5, 2007	February 9, 2007
Observations	February 8, 2007	February 8, 2007	February 12, 2007
Matrix Fortification	February 9, 2007	February 9, 2007	February 14, 2007
Definitive Test 454A-252A			
Observations	March 2, 2007	March 2, 2007	March 5, 2007
Matrix Fortification	March 2, 2007	March 2, 2007	March 5, 2007
Analytical Data, Biological Data and Draft Report	March 7 – 8, 2007	March 8, 2007	March 9, 2007
Final Report	March 14, 2007	March 14, 2007	March 15,2007

All inspections were study-based unless otherwise noted.

Linda R. Mitchell — Date: 15 March 2007

Linda R. Mitchell
Director of Regulatory and Ecotox Operations

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Lumbriculus variegatus*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-252A

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STUDY DIRECTOR:

Tui Minderhout, Ph.D.
Senior Biologist — March 15, 2007 Date

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SUMMARY

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Lumbriculus variegatus*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-252A

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TEST DATES:	Experimental Start:	February 26, 2007
	Biological Termination:	March 2, 2007
	Experimental Termination:	March 2, 2007

LENGTH OF EXPOSURE: 96 Hours

TEST ORGANISMS: Oligochaete (*Lumbriculus variegatus*)

SOURCE OF TEST ORGANISMS: Wildlife International, Ltd. Cultures
Easton, Maryland 21601

AGE OF TEST ORGANISMS: Adult at test start

TEST CONCENTRATIONS:	Nominal	Mean Measured
	Negative Control	< LOQ
	0.80 mg a.i./L	0.71 mg a.i./L
	1.5 mg a.i./L	1.4 mg a.i./L
	3.0 mg a.i./L	2.8 mg a.i./L
	6.0 mg a.i./L	5.6 mg a.i./L
	12 mg a.i./L	11 mg a.i./L

RESULTS: Based on mean measured concentrations:

96-Hour LC50:	5.6 mg a.i./L
95% Confidence Interval:	2.8 – 11 mg a.i./L
No-Mortality Concentration:	2.8 mg a.i./L
No-Observed-Effect Concentration:	2.8 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for 3M Corporation at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. An initial trial was conducted from February 5 to 9, 2007 but was repeated at lower concentrations to identify a no-observed-effect concentration (NOEC). The in-life phase of the definitive toxicity test was conducted from February 26 to March 2, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454A-252A in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the acute effects of perfluorooctanesulfonate, potassium salt (PFOS) on the oligochaete, *Lumbriculus variegatus*, during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Adult oligochaetes were exposed to a geometric series of five test concentrations and a negative control (dilution water) for 96 hours under static-renewal conditions. Ten replicate test chambers were maintained in each treatment and control group, with a single organism in each test chamber for a total of 10 oligochaetes per concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of exploratory range finding toxicity data and the initial trial. Nominal test concentrations selected were 0.80, 1.5, 3.0, 6.0 and 12 mg PFOS active ingredient (a.i.)/L. Test solutions were renewed at approximately 48 hours. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, prior to and following renewal at 48 hours, and at the end of the test.

Oligochaetes were impartially assigned to test chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 3, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups were used to determine LC50 values at 24, 48, 72 and 96 hours. The no-mortality concentration and the no-observed-effect concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Lumbriculus variegatus*". The protocol was based on procedures outlined in the U.S. Environmental Protection Agency Report number 600/R-99/064 *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates* (1) and ASTM Standard E729-96 *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates and Amphibians* (2).

Test Substance

The test substance used to prepare the test solutions, analytical calibration standards and the analytical matrix fortification samples for the study was received from 3M on October 29, 1998. It was assigned Wildlife International, Ltd. identification number 4675A upon receipt and was stored under ambient conditions. The test substance, a white powder, was identified as: FC-95, Lot number 217. The test substance contained 86.9% active ingredient and had an expiration date of August 31, 2016.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes 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. Adult oligochaetes used in the test were from Wildlife International, Ltd. cultures. The organisms were originally obtained from Environmental Consulting and Testing (ECT), Superior, Wisconsin. Prior to the test, the organisms were held in a container with paper toweling and overlying water from the same source and at approximately the same temperature as that used in the test. Oligochaetes were fed with a mixture of yeast, cereal grass media, and trout chow (YCT), during the holding time but were not fed during the test.

During the 2-week period immediately preceding the test, water temperatures in the holding container ranged from 23.3 to 24.4°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.0 to 8.4, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 7.2 to 7.9 mg/L (≥85% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter.

The organisms showed no signs of disease or stress during the holding period. At test initiation, oligochaetes were collected from the holding aquaria and indiscriminately transferred one at a time to each test chamber. All transfers were made below the water surface using wide-bore pipettes.

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 1.

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 was filtered to 0.45 μm to remove fine particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water are presented in Appendix 2.

Test Apparatus

Test chambers were 30-mL Nalgene® polypropylene plastic beakers filled with approximately 20 mL of water with no substrate provided. The depth of the test water in a representative chamber was 2.2 cm. Test chambers were positioned in a temperature-controlled chamber to maintain a temperature of 23 ± 1°C. Test chambers were covered with plastic wrap and were labeled with the project number, test concentration and replicate.

Preparation of Test Concentrations

A stock solution was prepared at a nominal concentration of 12 mg a.i./L, the highest concentration tested, by mixing a calculated amount of PFOS into dilution water (Wildlife International, Ltd. well water). The stock solution was mixed by sonication for 15 minutes followed by inversion. The stock appeared clear and colorless. Aliquots of the 12 mg a.i./L stock solution were proportionally diluted with well water to prepare 300 mL of test solution at nominal concentrations of 0.80, 1.5, 3.0 and 6.0 mg a.i./L. The solutions were mixed by inversion. All test solutions were adjusted to 100% active ingredient during preparation, based on the test substance

purity (86.9%). Test solutions were prepared for test initiation and renewal on day 2. All surviving oligochaetes were transferred from old to new solutions at approximately 48 hours. At test initiation and termination, all solutions appeared clear and colorless.

Analytical Sampling

At the beginning of the test and on day 2, samples were collected from the newly prepared batches of test solution to determine concentrations of the test substance. Prior to renewal at approximately 48 hours and at test termination, samples of test solution were collected from each test chamber and pooled by treatment group for analysis of test substance concentrations. All samples were collected at mid-depth, placed in plastic vials, and processed immediately for analysis.

Analytical Method

The analytical method used for the analysis of PFOS in freshwater was developed at Wildlife International, Ltd. The analytical method consisted of dilution of the samples 1:1, v/v with acetonitrile, followed by secondary dilution using acetonitrile: HPLC-grade bottled water (50:50, v/v), and analysis by direct injection high performance liquid chromatography with mass spectrometric (LC/MS/MS) detection.

Concentrations of PFOS in the samples were determined by LC/MS/MS using an Agilent 1100 Series High Performance Liquid Chromatograph interfaced with an Applied Biosystems / MDS Sciex API 3000 mass spectrometer (MS/MS) operated in negative ion multiple-reaction monitoring (MRM) detection mode. The mass spectrometer was equipped with a TurboIon Spray ion source. Chromatographic separations were achieved using a Zorbax RX-C_8 column (150 mm × 2.1 mm, 5µm particle size). A flow chart for the analysis of PFOS is provided in Appendix 3.1 and typical instrumental parameters are summarized in Appendix 3.2.

Calibration standards of PFOS, ranging in concentration from 0.0500 to 1.00 µg a.i./mL, were prepared in acetonitrile: HPLC-grade bottled water solution (50:50, v/v) using a stock solution of PFOS in methanol (Appendix 3.3). Quadratic (weighted 1/x) regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards using Analyst Version 1.4.1 software of the Applied Biosystems/MDS Sciex API 3000 mass spectrometer system. The concentration of PFOS in the samples was determined by substituting the peak area

responses of the samples into the applicable regression equation. An example of the calculations for a representative sample is included in Appendix 3.4.

The method limit of quantitation (LOQ) for these analyses was set at 0.200 mg a.i./L, calculated as the product of the lowest calibration standard (0.0500 µg a.i./mL) and the dilution factor of the matrix blank samples (4.00). Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 3.5).

Matrix fortification samples were prepared fresh on each sampling day and were analyzed concurrently with the samples. Samples of freshwater were fortified with a stock solution of the test substance in methanol at nominal PFOS concentrations of 0.500, 3.00 and 15.0 mg a.i./L. The measured concentrations for the matrix fortification samples ranged from 96.4 to 108% of nominal concentrations (Appendix 3.5).

A representative calibration curve is presented in Appendix 3.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 3.9 and a representative chromatogram of a matrix fortification sample is presented in Appendix 3.10. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the cultures and test chambers. 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, measured using a SPER Scientific Model 840006C light meter, was 648 lux at the surface of the water of one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in two alternate replicates at the beginning of the test, prior to and after each renewal (old and new solutions) and at the end of the test (old solution) using a liquid-in-glass thermometer. Temperature was also

monitored daily in a container of water adjacent to the test chambers in the environmental chamber using a continuous temperature recorder.

Dissolved oxygen and pH were measured in samples collected from batches of new test solutions or were measured in composite samples of old solutions. Old solutions from the replicates of each test group were combined and a composite sample collected for analysis. Dissolved oxygen was measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, and measurements of pH were made using a Thermo Orion Model 525Aplus meter.

Hardness, alkalinity and specific conductance were measured in the dilution water at test initiation and in the composite of negative control at test termination. 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* (3).

Observations

Observations of mortality were made periodically in each treatment group. Lethality is defined as the lack of visible movement in the oligochaete. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 3, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The mortality data were analyzed using the computer program of C. E. Stephan (4). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (5, 6 and 7). In this study, the binomial method was used to calculate the 24, 48, 72 and 96-hour LC50 values. The no-mortality concentration and NOEC were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations selected for use in this study were 0.80, 1.5, 3.0, 6.0 and 12 mg a.i./L. Results of analyses to measure concentrations of PFOS in the test solution samples collected during the test are presented in Table 1. Samples collected at test initiation had measured concentrations that ranged from 92 to 97% of the nominal concentrations. Samples collected prior to and after renewal of the test solutions on day 2 had measured concentrations that ranged from 88 to 93% and 85 to 94%, respectively, of the nominal concentrations. Samples collected at test termination had measured concentrations that ranged from 88 to 97% of the nominal concentrations. When measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations for this study were 0.71, 1.4, 2.8, 5.6 and 11 mg a.i./L, representing 89, 93, 93, 93 and 92% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the water in the test chambers are presented in Table 2. Water temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations remained ≥7.4 mg/L (≥87% of saturation) throughout the test. Measurements of pH ranged from 8.2 to 8.6. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation and termination were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations for mortality and signs of toxicity during the test are presented in Table 4. Oligochaetes in the negative control group appeared normal throughout the test. All oligochaetes in the 0.71, 1.4 and 2.8 mg a.i./L treatment groups also appeared normal throughout the test, with no mortality or overt signs of toxicity observed. Percent mortality at test termination in the 5.6 and 11 mg a.i./L treatment groups was 50 and 100%, respectively. The no-mortality concentration and the NOEC were both 2.8 mg a.i./L. LC50 values at 24, 48, 72 and 96 hours were determined from the mortality data and are shown in Table 5. A graph of the concentration-response curve is included in Figure 1.

CONCLUSIONS

The oligochaete, *Lumbriculus variegatus*, was exposed for 96 hours under static-renewal conditions to five mean measured concentrations of PFOS ranging from 0.71 to 11 mg a.i./L. The 96-hour LC50 value was 5.6 mg a.i./L, with a 95% confidence interval of 2.8 to 11 mg a.i./L. The no-mortality concentration and the NOEC were both 2.8 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates.* EPA 600/R-99/064.

2 **ASTM Standard E729-96.** 1996. *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

3 **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.

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

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

6 **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.

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

Table 1

Measured Concentrations of PFOS in Freshwater Samples

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-252A-)	Sampling Time (Hours)	Measured Concentration PFOS (mg a.i./L) [1]	Percent of Nominal [1]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
0.00 (Negative Control)	1	0(new)	< LOQ[2]	--	< LOQ	--
	7	48(new)	< LOQ	--		
	13	48(old)	< LOQ	--		
	19	96(old)	< LOQ	--		
0.80	2	0(new)	0.749	93.7	0.71	88.8
	8	48(new)	0.683	85.4		
	14	48(old)	0.707	88.4		
	20	96(old)	0.700	87.5		
1.5	3	0(new)	1.37	91.6	1.4	93.3
	9	48(new)	1.39	92.4		
	15	48(old)	1.35	90.1		
	21	96(old)	1.36	90.5		
3.0	4	0(new)	2.87	95.7	2.8	93.3
	10	48(new)	2.73	91.0		
	16	48(old)	2.80	93.3		
	22	96(old)	2.88	95.9		
6.0	5	0(new)	5.80	96.7	5.6	93.3
	11	48(new)	5.64	94.0		
	17	48(old)	5.33	88.9		
	23	96(old)	5.80	96.7		
12	6	0(new)	11.4	94.9	11	91.7
	12	48(new)	11.3	93.8		
	18	48(old)	11.0	91.5		
	24	96(old)	11.6	96.8		

[1] Results generated using Analyst version 1.4.1 software. Manual calculations may vary.

[2] The limit of quantitation (LOQ) was 0.200 mg a.i./L calculated as the product of the lowest calibration standard (0.0500 µg a.i./mL) and the dilution factor of the matrix blanks (4.00).

Table 2

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

Mean Measured Concentration (mg a.i./L)	0 Hour			48 Hours (Old Solution Prior to Renewal)			48 Hours (New Solution At Renewal)			96 Hours		
	Temp.[1] (°C)	DO[2] (mg/L)	pH[2]	Temp.[1] (°C)	DO[2] (mg/L)	pH[2]	Temp.[1] (°C)	DO[2,] (mg/L)	pH[2]	Temp.[1] (°C)	DO[2] (mg/L)	pH[2]
Negative Control	22.4	8.3	8.2	23.8	8.0	8.6	23.4	8.2	8.2	23.3	8.3	8.6
	22.4	--	--	23.8	--	--	23.8	--	--	23.3	--	--
0.71	22.9	8.3	8.2	23.8	8.0	8.6	23.1	8.4	8.3	23.5	8.3	8.6
	22.7	--	--	23.9	--	--	23.2	--	--	23.5	--	--
1.4	22.9	8.4	8.2	23.9	8.0	8.6	23.0	8.4	8.3	23.4	8.4	8.6
	22.9	--	--	23.8	--	--	22.8	--	--	23.5	--	--
2.8	23.2	8.4	8.2	23.8	8.0	8.6	22.5	8.3	8.3	23.4	8.3	8.6
	22.7	--	--	24.0	--	--	22.5	--	--	23.7	--	--
5.6	23.2	8.4	8.2	24.0	7.9	8.5	22.8	8.2	8.3	23.5	8.3	8.6
	23.2	--	--	23.8	--	--	23.1	--	--	23.6[4]	--	--
11	23.1	8.3	8.2	23.5	7.4	8.5	23.1[3]	8.2	8.2	23.1[5]	8.2	8.6
	22.9	--	--	23.5	--	--	23.2[3]	--	--	23.3[5]	--	--

[1] Manual temperature measurements replicate: day 0 (A, B); 48-hr old (C, D); 48-hr new (E, F); 96-hr old (G, H). Temperature measured continuously during the test ranged from 22.9 – 24.3°C.

[2] Dissolved oxygen and pH were measured in samples collected from batches of new test solution or measured in composite samples of old solutions. A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C in freshwater.

[3] Manual temperature measurements were taken at 48 hours in replicates H and I due to mortality in replicate C.

[4] Manual temperature measurements were taken at 96 hours in replicate I due to 100% mortality in replicate H.

[5] Manual temperature measurements were taken at 96 hours in replicate D and H due to 100% mortality in replicate G.

Table 3

Specific Conductance, Hardness and Alkalinity
Measured in Dilution Water at Test Initiation and
in Composite of Negative Control at Test Termination

Parameter	Day 0	Day 4
Specific Conductance (µmhos/cm)	300	320
Hardness (mg/L as $CaCO_3$)	128	138
Alkalinity (mg/L as $CaCO_3$)	180	190

Table 4

Cumulative Mortality and Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	3 Hours		24 Hours		48 Hours (T)[4]		72 Hours		96 Hours		Cumulative Percent Mortality
		No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	
Negative Control	A	0	AN	0	AN	0	AN	0	AN	0	AN	0
	B	0	AN	0	AN	0	AN	0	AN	0	AN	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	0	AN	0	AN	
	E	0	AN	0	AN	0	AN	0	AN	0	AN	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	0	AN	0	AN	0	AN	0	AN	0	AN	
	H	0	AN	0	AN	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	
0.71	A	0	AN	0	AN	0	AN	0	AN	0	AN	0
	B	0	AN	0	AN	0	AN	0	AN	0	AN	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	0	AN	0	AN	
	E	0	AN	0	AN	0	AN	0	AN	0	AN	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	0	AN	0	AN	0	AN	0	AN	0	AN	
	H	0	AN	0	AN	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	

[1] A single midge was exposed in each replicate.
[2] Cumulative number of dead midges.
[3] Observations: AN = appear normal.
[4] T = transferred to new test solution.

Table 4 (Continued)

Cumulative Mortality and Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	3 Hours		24 Hours		48 Hours (T)[4]		72 Hours		96 Hours		Cumulative Percent Mortality
		No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	
1.4	A	0	AN	0	AN	0	AN	0	AN	0	AN	0
	B	0	AN	0	AN	0	AN	0	AN	0	AN	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	0	AN	0	AN	
	E	0	AN	0	AN	0	AN	0	AN	0	AN	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	0	AN	0	AN	0	AN	0	AN	0	AN	
	H	0	AN	0	AN	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	
2.8	A	0	AN	0	AN	0	AN	0	AN	0	AN	0
	B	0	AN	0	AN	0	AN	0	AN	0	AN	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	0	AN	0	AN	
	E	0	AN	0	AN	0	AN	0	AN	0	AN	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	0	AN	0	AN	0	AN	0	AN	0	AN	
	H	0	AN	0	AN	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	

[1] A single midge was exposed in each replicate.
[2] Cumulative number of dead midges.
[3] Observations: AN = appear normal.
[4] T = transferred to new test solution.

Table 4 (Continued)

Cumulative Mortality and Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	3 Hours		24 Hours		48 Hours (T)[4]		72 Hours		96 Hours		Cumulative Percent Mortality
		No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	No. Dead[2]	Obs.[3]	
5.6	A	0	AN	0	AN	0	AN	0	D	0	D	50
	B	0	AN	0	D, W	1	--	1	--	1	--	
	C	0	AN	0	AN	0	AN	0	D	0	D	
	D	0	AN	0	D	1	--	1	--	1	--	
	E	0	AN	0	AN	0	D	0	D	1	--	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	0	AN	0	AN	0	AN	0	AN	0	AN	
	H	0	AN	0	AN	1	--	1	--	1	--	
	I	0	AN	0	AN	0	AN	0	D	0	D	
	J	0	AN	0	AN	0	AN	0	AN	1	--	
11	A	0	D	1	--	1	--	1	--	1	--	100
	B	0	D	1	--	1	--	1	--	1	--	
	C	0	W	0	D, W	1	--	1	--	1	--	
	D	0	AN	0	AN	0	D	1	--	1	--	
	E	0	D	1	--	1	--	1	--	1	--	
	F	0	D, W	1	--	1	--	1	--	1	--	
	G	0	D	0	D	1	--	1	--	1	--	
	H	0	D	0	D	0	D	1	--	1	--	
	I	0	AN	0	AN	0	D	1	--	1	--	
	J	0	D, W	1	--	1	--	1	--	1	--	

[1] A single midge was exposed in each replicate.
[2] Cumulative number of dead midges.
[3] Observations: AN = appear normal, D = pale, W = fragmentation.
[4] T = transferred to new test solution.

Table 5

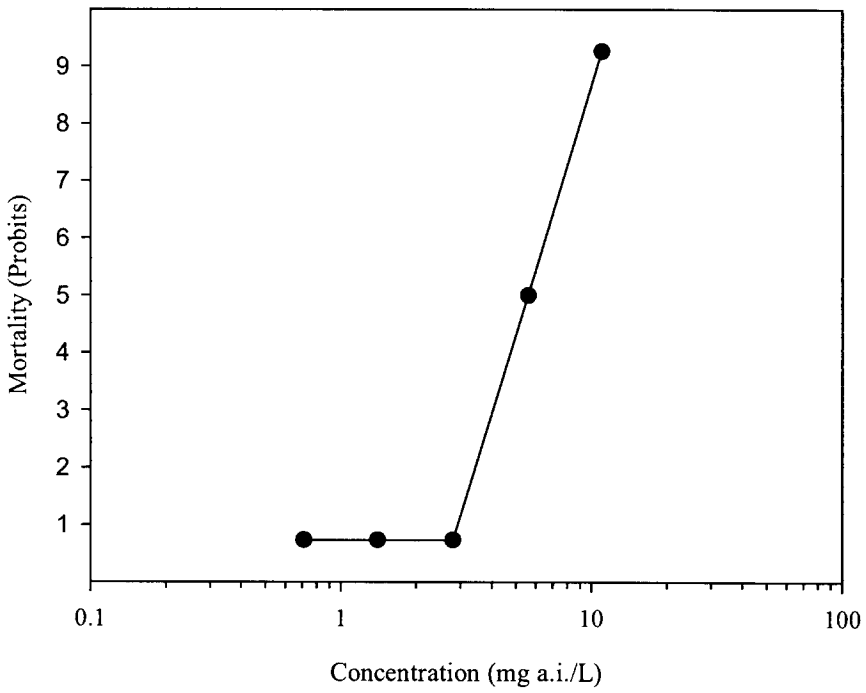
LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	11	>5.6[1]	Binomial Probability
48 Hours	7.9	>2.8[2]	Binomial Probability
72 Hours	6.5	2.8 – 11	Binomial Probability
96 Hours	5.6	2.8 – 11	Binomial Probability

[1] At a confidence level of 95% the binomial test shows that the LC50 is above 5.6 mg a.i./L.
[2] At a confidence level of 95% the binomial test shows that the LC50 is above 2.8 mg a.i./L.

Figure 1

Concentration-Response Curve (96-Hour Mortality Data)



Appendix 1

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

Parameter	Mean	Range
Specific Conductance (µmhos/cm)	291 (N = 4)	285 – 300
Hardness (mg/L as $CaCO_3$)	136 (N = 4)	132 – 140
Alkalinity (mg/L as $CaCO_3$)	182 (N = 4)	180 – 184
pH	8.1 (N = 4)	7.9 – 8.1

Appendix 2

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.019	Heptachlor	< 0.0096
Alpha BHC	< 0.0096	Heptachlor Epoxide	< 0.0096
Alpha Chlordane	< 0.0096	Kepone	< 0.19
Beta BHC	< 0.038	Malathion	< 1.9
Bolstar	< 1.9	Merphos	< 1.9
Chlordane	< 0.48	Methoxychlor	< 0.096
Coumaphos	< 2.9	Methyl Parathion	< 1.9
Delta BHC	< 0.0096	Mevinphos	< 1.9
Demeton-O	< 1.9	Mirex	< 0.11
Demeton-S	< 1.9	Naled	< 2.9
Diazinon	< 1.9	o,p-DDD	< 0.019
Dichlorvos	< 1.9	o,p-DDE	< 0.019
Dieldrin	< 0.029	o,p-DDT	< 0.019
Disulfoton	< 1.9	p,p-DDD	< 0.019
Dursban (Chlorpyrifos)	< 1.9	p,p-DDE	< 0.019
Endosulfan I	< 0.0096	p,p-DDT	< 0.019
Endosulfan II	< 0.019	PCB-1016	< 0.48
Endosulfan Sulfate	< 0.019	PCB-1221	< 0.48
Endrin	< 0.019	PCB-1232	< 0.48
Endrin Aldehyde	< 0.096	PCB-1242	< 0.48
Endrin Ketone	< 0.019	PCB-1248	< 0.48
EPN	< 3.8	PCB-1254	< 0.48
Ethion	< 1.9	PCB-1260	< 0.48
Ethoprop	< 1.9	Phorate	< 1.9
Ethyl Parathion	< 1.9	Ronnel	< 1.9
Famphur	< 1.9	Stirophos	< 1.9
Fensulfothion	< 3.8	Telodrin	< 0.0096
Fenthion	< 1.9	Tokuthion	< 1.9
Gamma BHC – Lindane	< 0.0096	Toxaphene	< 0.96
Gamma Chlordane	< 0.096	Trichloronate	< 1.9
Guthion (Azinphos-methyl)	< 3.8	Trithion	< 1.9
HCB	< 0.096		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 15, 2005.

Appendix 2 (Continued)

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

Metals			
Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum	< 0.200	Magnesium	13.3
Antimony	< 0.0200	Manganese	< 0.0050
Arsenic	< 0.0200	Mercury	< 0.00020
Barium	< 0.0050	Nickel	< 0.0100
Beryllium	< 0.0050	Nitrate Nitrogen	< 0.50
Bromide	< 2.5	Nitrite Nitrogen	< 0.50
Cadmium	< 0.0050	Potassium	7.65
Calcium	33.1	Selenium	< 0.0200
Chloride	2.7	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.1
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	0.56	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0200		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 15, 2005.

Appendix 3

The Analysis of PFOS in Freshwater

Appendix 3.1

Analytical Method Flowchart for the Processing of PFOS in Freshwater

METHOD OUTLINE FOR THE ANALYSIS OF PFOS IN FRESHWATER

Prepare calibration standards in acetonitrile : HPLC-grade bottled water (50:50,v/v) using volumetric flasks and gas-tight syringes, STORE REFRIGERATED.

↓

Prepare matrix fortification samples in well water using volumetric flasks, volumetric pipettes, 15-mL culture tubes and gas-tight syringes.

↓

Dilute **all** samples initially 1:1 with 100% acetonitrile using 15-mL culture tubes or equivalent, gas-tight syringes and/or class A volumetric pipettes. Mix well. Volumetrically dilute solutions further, if necessary, with acetonitrile : HPLC-grade bottled water (50:50,v/v) so that the final sample concentrations fall within the calibration standard range. Mix well.

↓

Transfer aliquots of final sample dilutions and calibration standards to autosampler vials for analysis by LC/MS/MS.

↓

Appendix 3.2

Typical HPLC/MS/MS Operational Parameters

INSTRUMENT:	Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) coupled with an Applied Biosystems/MDS Sciex API 3000 Mass Spectrometer (MS/MS) operated in the negative ion multiple-reaction monitoring (MRM) mode.
ION SOURCE:	TurboIon Spray
ANALYTICAL COLUMN:	Zorbax RX-C_8 (150 mm x 2.1 mm, 5 µm particle size)
STOP TIME:	5.00 minutes
FLOW RATE:	0.300 mL/minute
OVEN TEMPERATURE:	40°C
MOBILE PHASE:	80% CH_3OH : 20% H_2O containing 0.1% formic acid
INJECTION VOLUME:	10.0 µL
PFOS RETENTION TIME:	Approximately 2.8 minutes
PFOS MONITORED MASS:	499 → 99 amu

Appendix 3.3

Analytical Stocks and Standards Preparation

A stock solution of PFOS was prepared by weighing 1.1507 g (corrected for purity) of the test substance on an analytical balance. The test substance was transferred to a 100-mL volumetric flask and brought to volume using methanol. This primary stock solution contained 10.0 mg a.i./mL of PFOS. Secondary stocks of PFOS in methanol (1.00 and 0.100 mg a.i./mL) were prepared from the primary stock by volumetric dilution. The 1.00 mg a.i./mL secondary stock solution was used to prepare concurrent matrix fortification samples (QC) for this study. The 0.100 mg a.i./mL secondary stock solution was used to prepare calibration standards. The calibration standards were prepared in acetonitrile: HPLC-grade bottled water (50:50, v/v). The following shows the dilution scheme for the set of calibration standards.

Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (mL)	Standard Concentration (µg a.i./mL)
0.100	0.0500	100	0.0500
0.100	0.150	100	0.150
0.100	0.250	100	0.250
0.100	0.500	100	0.500
0.100	1.00	100	1.00

Appendix 3.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 454A-252A-2, an exposure sample prepared at a nominal concentration of 0.80 mg a.i./L, was calculated as follows using the software algorithms of Analyst Version 1.4.1 of the Applied Biosystems/MDS Sciex API 3000 mass spectrometer system. Regression was used to generate calibration equations for each analytical sequence relating the measured peak areas of reference standard solution injections of PFOS with their known concentrations. The curve was weighted 1/x with respect to concentration and expressed as a quadratic function as follows:

$y = ax^2 + bx + c$

where y = instrumental peak area response of concentration x of PFOS in mg a.i./L
a = quadratic coefficient
b = linear coefficient
c =constant coefficient (y_intercept)

Concentrations of PFOS in samples were determined by substituting peak area responses of the samples into the applicable rearranged regression equation as follows:

PFOS (mg a.i./L) = Dilution Factor •

$$\frac{-\text{Linear Coefficient} + \sqrt{(\text{Linear Coefficien t})^2 - [4 \bullet (\text{Quadratic Coefficien t}) \bullet (\text{Y\_Interce pt} - \text{Peak Area})]}}{2 \bullet (\text{Quadratic Coefficien t})}$$

where the Dilution Factor compensates for dilution of the water sample so that the peak response was bracketed by the standard calibration curve.

Data used for quantitation of PFOS in Sample Number 454A-252A-2 are summarized below:

Peak area = 1066000
Constant Coefficient = -6561.21
Linear Coefficient = 5757530
Quadratic Coefficient = -174737
Dilution Factor ($V_{final}/V_{initial}$): = 4.00

Appendix 3.4 (Continued)

Example Calculations for a Representative Sample

$$PFOS = 4.00 \bullet \frac{-5757530 + \sqrt{(5757530)^2 - [(4 \bullet (-174737)) \bullet (-6561.21 - 1066000)]}}{2 \bullet (-174737)}$$

PFOS = 4.00 • 0.18735 mg a.i./L

PFOS = 0.749 mg a.i./L

The measured concentration was compared to the nominal concentrations as follows:

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

$$= \frac{0.749 \text{ mg/L}}{0.800 \text{ mg/L}} \text{ X } 100$$

= 93.7%

Appendix 3.5

Quality Control Samples of PFOS in Freshwater

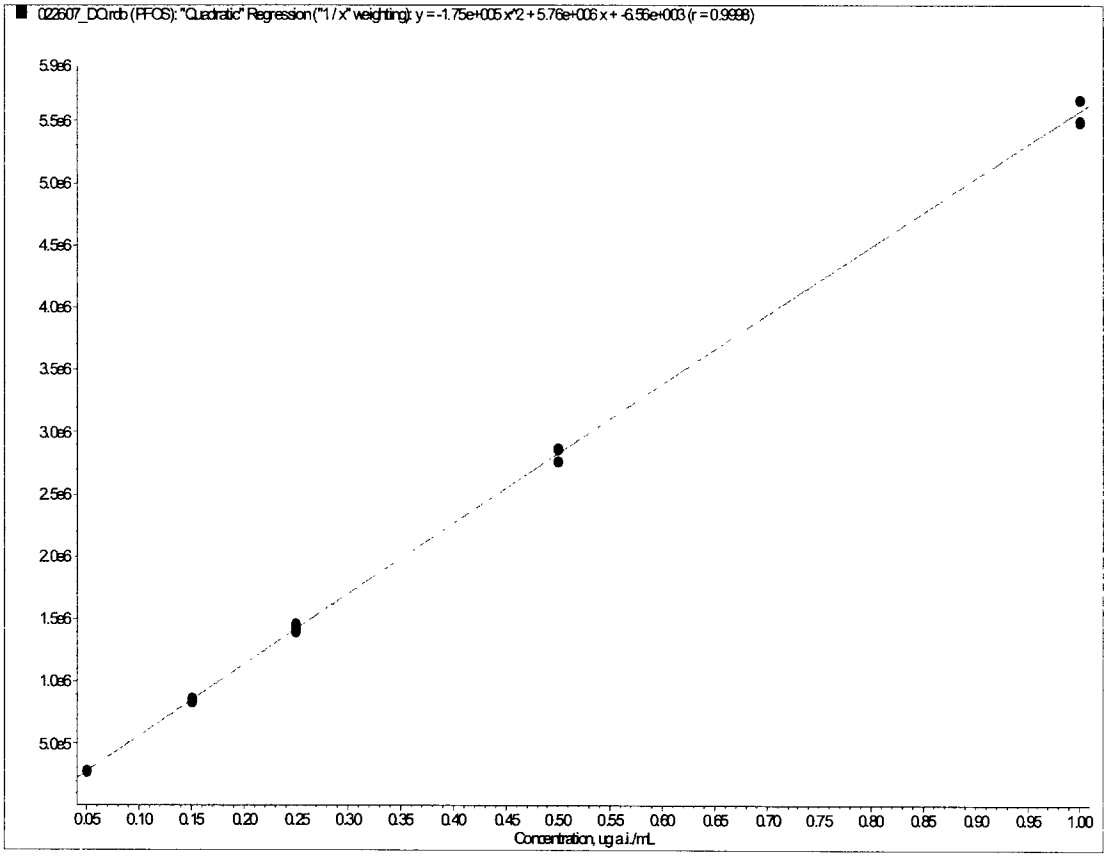
Sample Number (454A-252A-)	Sampling Time (Hours)	Concentration (mg a.i./L)		Percent Recovery [1]
		Fortified	Measured [1,2]	
MAB-1	0	0.0	< LOQ	--
MAB-2	48	0.0	< LOQ	--
MAB-3	96	0.0	< LOQ	--
MAS-1	0	0.500	0.540	108
MAS-2	0	3.00	3.11	104
MAS-3	0	15.0	14.9	99.3
MAS-4	48	0.500	0.486	97.2
MAS-5	48	3.00	3.01	100
MAS-6	48	15.0	14.5	96.4
MAS-7	96	0.500	0.490	98.0
MAS-8	96	3.00	3.07	102
MAS-9	96	15.0	14.6	97.3
				X=100 S.D.=3.81 C.V.=3.81%

[1] Results generated using Analyst version 1.4.1 software. Manual calculations may vary.

[2] The limit of quantitation (LOQ) was 0.200 mg a.i./L calculated as the product of the lowest calibration standard (0.0500 µg a.i./mL) and the dilution factor of the matrix blanks (4.00).

Appendix 3.6

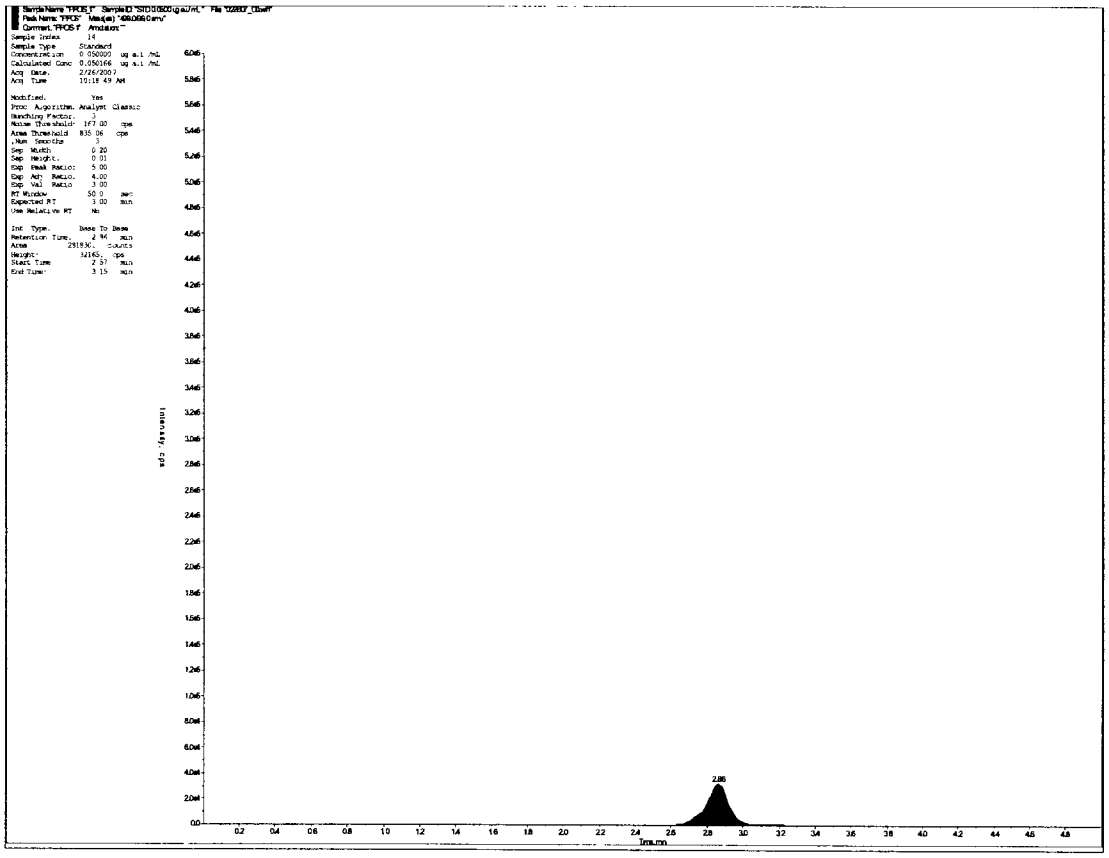
Representative Calibration Curve for PFOS



Linear coefficient=5757530; constant coefficient=-6561.21; quadratic coefficient= -174737; r=0.9998

Appendix 3.7

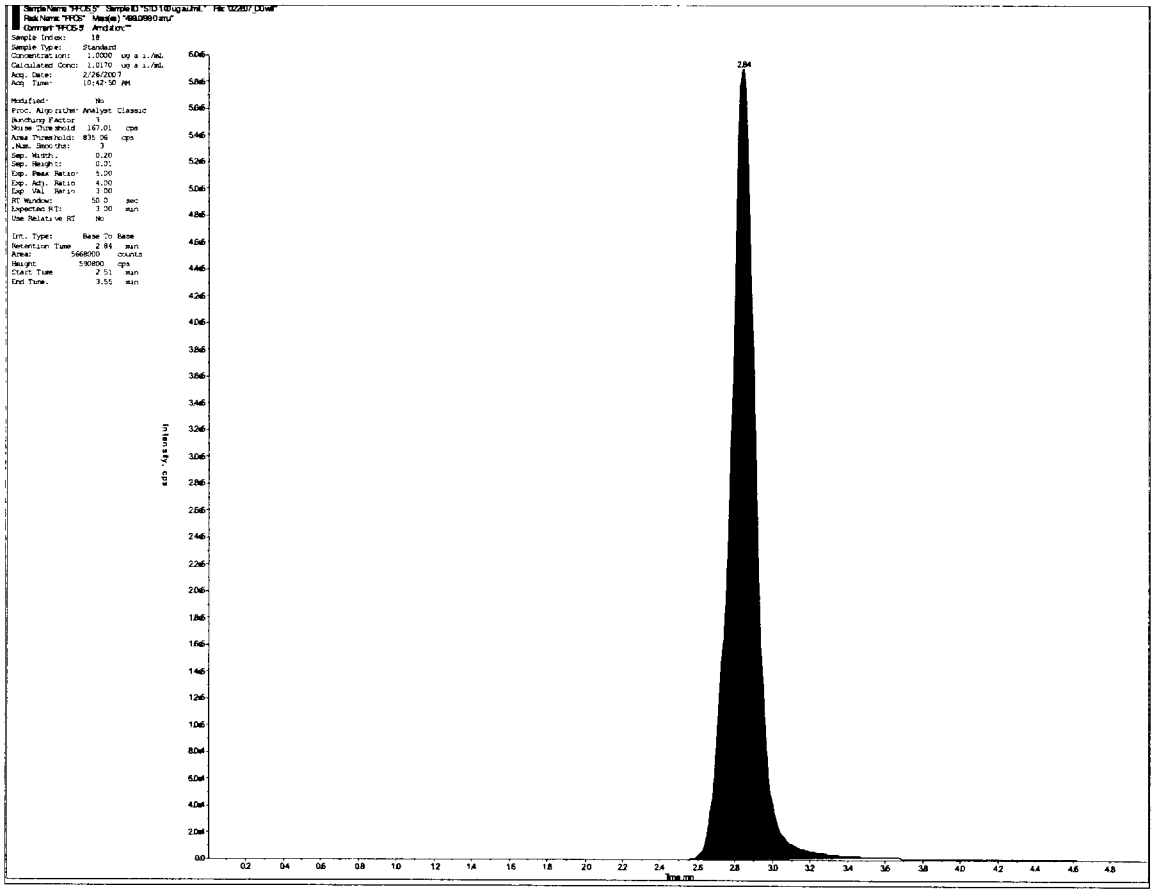
Representative Chromatogram of a Low-level PFOS Calibration Standard



Nominal concentration: 0.0500 mg a.i./L

Appendix 3.8

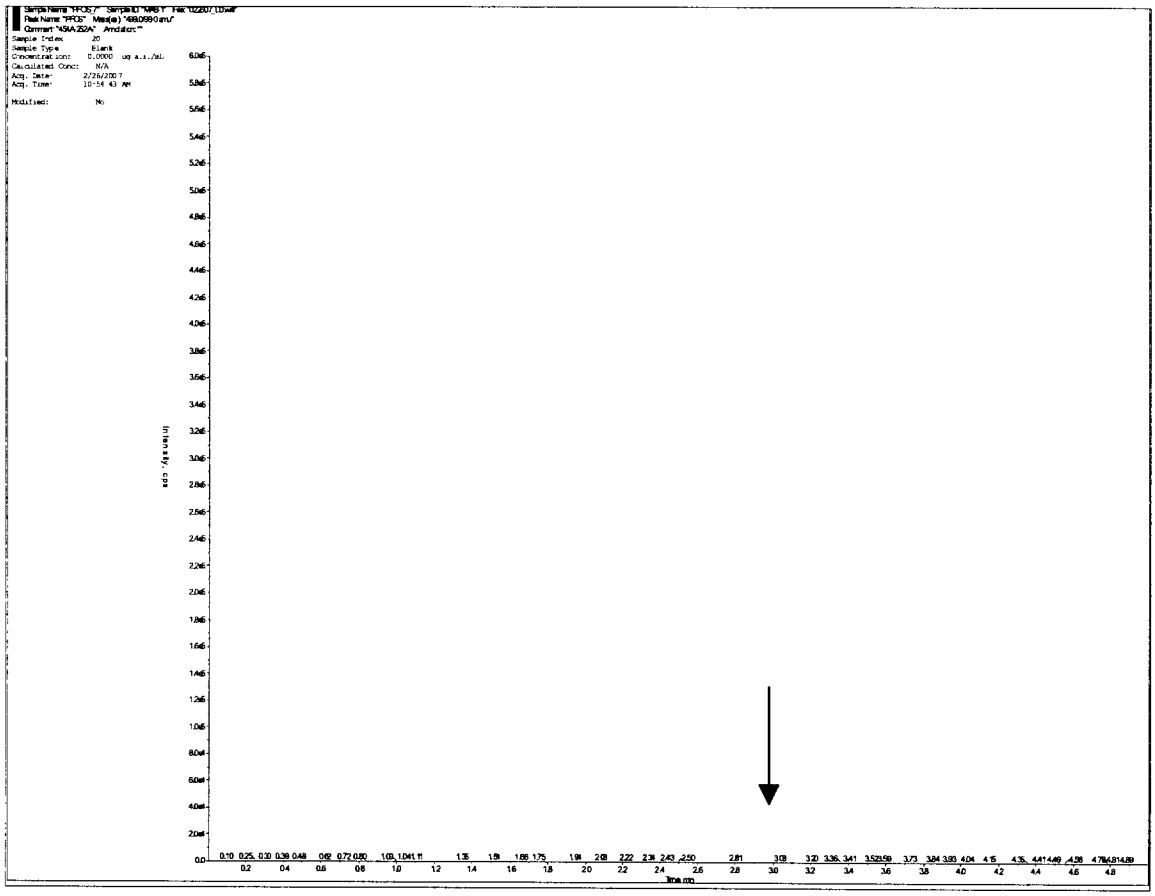
Representative Chromatogram of a High-level PFOS Calibration Standard



Nominal concentration: 1.00 mg a.i./L

Appendix 3.9

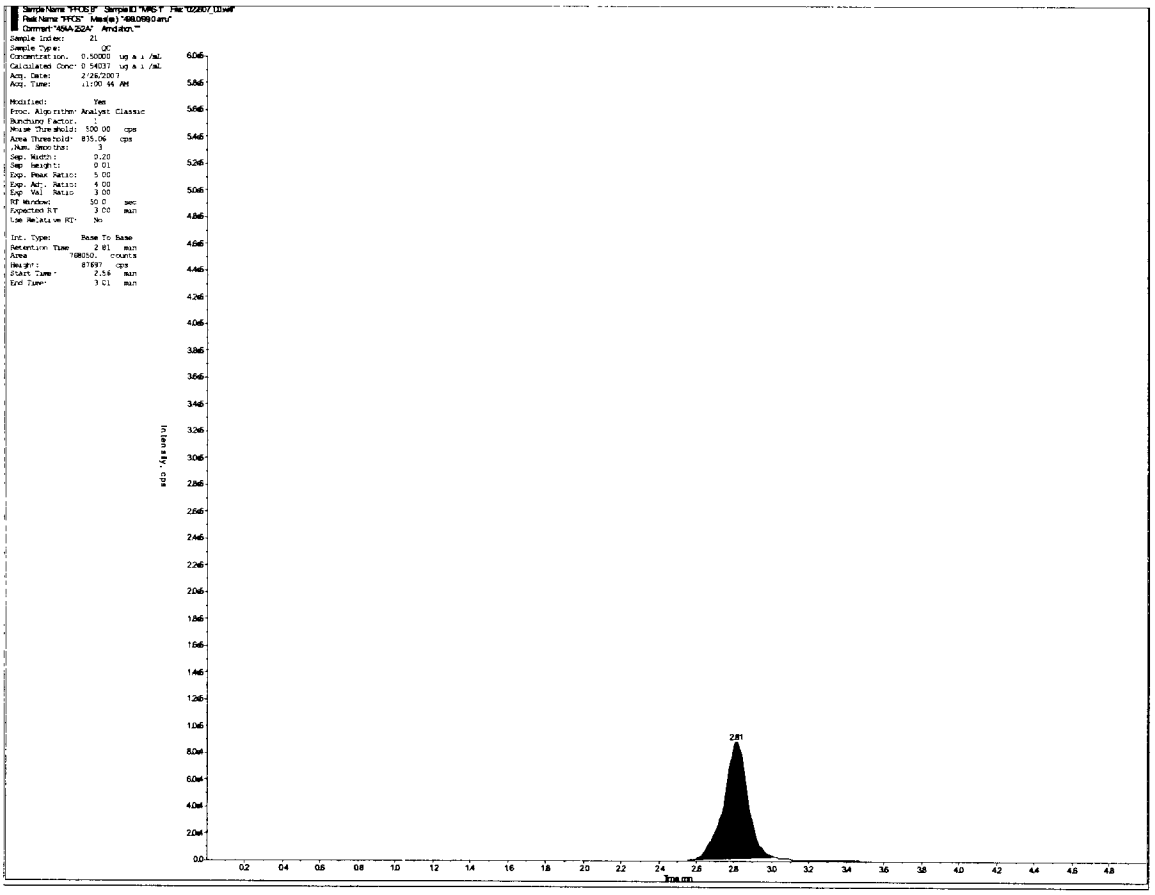
Representative Chromatogram of a Matrix Blank Sample



Sample number 454A-252A-MAB-1. Dilution factor = 4.00X. The arrow indicates the retention time of PFOS.

Appendix 3.10

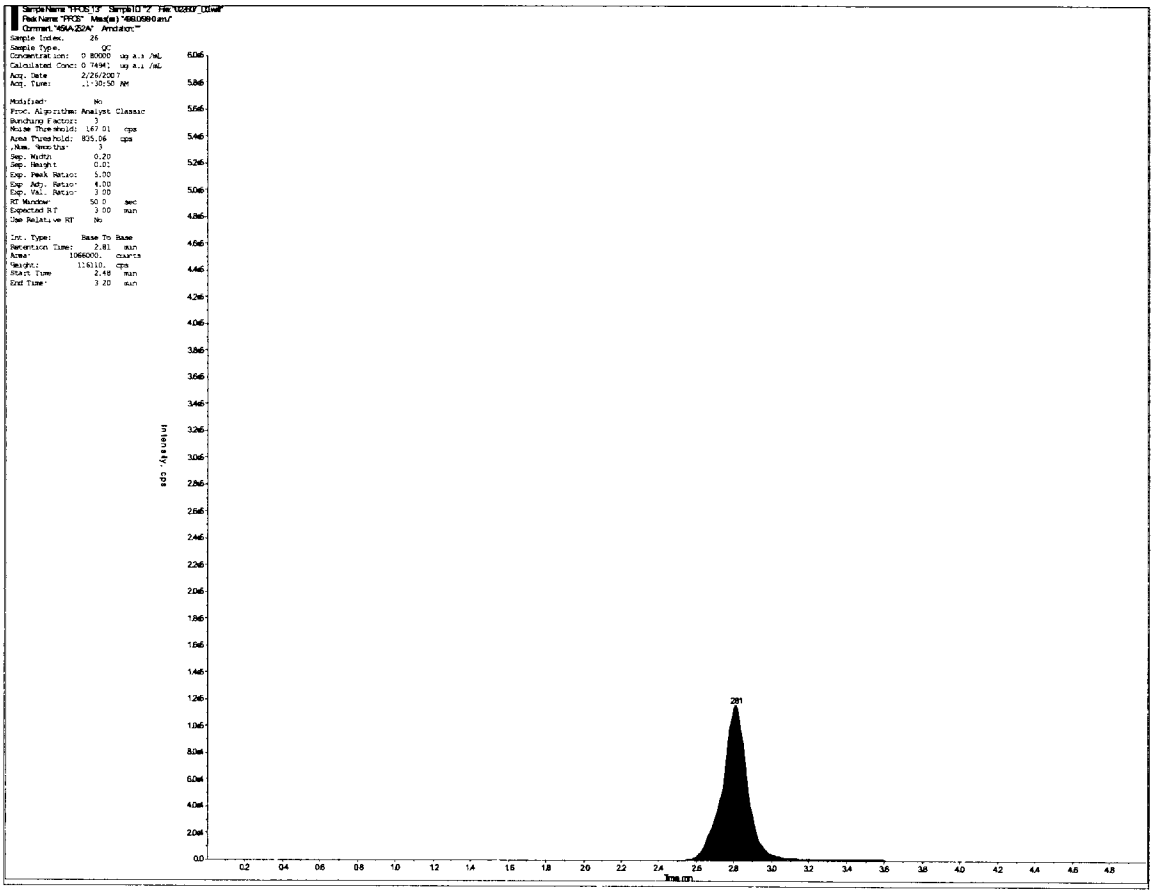
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 454A-252A-MAS-1, nominal concentration 0.500 mg/L. Dilution factor = 4.00X.

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 454A-252A-2, Day 0, nominal concentration 0.80 mg a.i./L. Dilution factor = 4.00X.

Appendix 4

Changes to Protocol

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

1. The protocol was amended to include measurement of the temperature in two alternate replicates, rather than in each replicate, at the beginning of the test, prior to and after each renewal (old and new solutions) and at the end of the test (old solution) using a liquid-in-glass thermometer.

2. The protocol was amended to include monitoring of the temperature daily in a container of water adjacent to the test chambers in the environmental chambers using a continuous temperature recorder, rather than in the negative control chamber.

3. The protocol was amended to include measurement of dissolved oxygen and pH in samples collected from batches of new test solutions or measured in composite samples of old solutions. Old solutions from the replicates of each test group were combined and a composite sample collected for analysis.

4. The protocol was amended to include the Environmental Laboratory Project Number of E07-0084.

5. The protocol was amended to change the test concentrations for the repeat test to 0.80, 1.5, 3.0, 6.0 and 12 mg a.i./L.

6. The test organisms were held in a container with paper toweling and overlying water, rather than in container with sand and overlying water. During the holding period test organisms were fed with a mixture of YCT only without the supplement of green algae. This had no adverse impact on the study results.

Appendix 5

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 of Aquatic Toxicology/Terrestrial Plants and Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Tui Minderhout, Ph.D., Senior Biologist
4. Amy S. Blankinship, Laboratory Supervisor, Aquatics
5. Jon A. MacGregor, Scientist