PFOS: A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH *Hyalella azteca*

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-253A
3M ENVIRONMENTAL LABORATORY PROJECT NUMBER: E07-0082

ASTM Standard E729-96

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STUDY INITIATION DATE: February 15, 2007

STUDY COMPLETION DATE: March 29, 2007

SUBMITTED TO:

3M Corporation
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Hyalella azteca*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-253A

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STUDY COMPLETION: March 29, 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 exception:

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

STUDY DIRECTOR:

B Tui Minderhout — 3/29/2007

Tui Minderhout, Ph D. — Date
Senior Biologist

SPONSOR APPROVAL:

John Newsted — 4/3/2007

Sponsor Representative — 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	February 19, 2007	February 19, 2007	February 20, 2007
Initial Trial: 454A-253A			
Test Substance Preparation	February 16, 2007	February 16, 2007	February 21, 2007
Definitive Test: 454A-253A			
Matrix Fortification	March 9, 2007	March 9, 2007	March 15, 2007
Observations	March 9, 2007	March 9, 2007	March 15, 2007
Analytical Data and Draft Report	March 20 and 21, 2007	March 21, 2007	March 22, 2007
Biological Data and Draft Report	March 19 – 21, 2007	March 21, 2007	March 26, 2007
Final Report	March 29, 2007	March 29, 2007	March 29, 2007

All inspections were study-based unless otherwise noted.

James H. Coleman

James H. Coleman
Quality Assurance Representative

3-29-07

Date

REPORT APPROVAL

SPONSOR: 3M Corporation

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SUMMARY

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with *Hyalella azteca*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-253A

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TEST DATES:	Experimental Start:	March 5, 2007
	Biological Termination:	March 9, 2007
	Experimental Termination:	March 9, 2007

LENGTH OF EXPOSURE: 96 Hours

TEST ORGANISMS: Amphipod (*Hyalella azteca*)

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
Superior, Wisconsin

AGE OF TEST ORGANISMS: 12 days old at test initiation

TEST CONCENTRATIONS:	Nominal	Mean Measured
	Negative Control	< LOQ
	13 mg a.i./L	13 mg a.i./L
	25 mg a.i./L	25 mg a.i./L
	50 mg a.i./L	51 mg a.i./L
	100 mg a.i./L	102 mg a.i./L
	200 mg a.i./L	199 mg a.i./L

RESULTS: Based on mean measured concentrations:

96-Hour LC50:	15 mg a.i./L
95% Confidence Interval:	4.5 – 24 mg a.i./L
No-Mortality Concentration:	<13 mg a.i./L
No-Observed-Effect Concentration:	<13 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 19 to 22, 2007 but was terminated due to unacceptable percent survival in the negative control possibly caused by stress of organisms during shipment. The in-life phase of the definitive toxicity test was conducted from March 5 to 9, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454A-253A 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 amphipod, *Hyalella azteca*, during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Amphipods 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 groups while twenty replicates were maintained in the control group. A single organism was placed in each test chamber for a total of 10 amphipods per treatment concentration and twenty amphipods per negative control. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of exploratory range finding toxicity data. Nominal test concentrations selected were 13, 25, 50, 100 and 200 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 renewal at 48 hours, and at the end of the test.

Amphipods were impartially assigned to test chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 4.5, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was 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 *Hyalella azteca*". 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 amphipod, *Hyalella azteca*, was selected as the test species for this study. Amphipods 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. Amphipods used in the test were obtained from Environmental Consulting and Testing (ECT), Superior, Wisconsin. Amphipods were hatched on February 21, 2007 and were 12 days old at test initiation. Prior to the test, the organisms were held for 6 days in a container with pieces of gauze and overlying water from the same source and at approximately the same temperature as that used in the test. Amphipods were fed YCT (1.8 g/L) and Tetramin® flake food during the holding period and YCT on days 0 and 2 of the test.

During the 6 days immediately preceding the test, water temperatures in the holding container ranged from 21.8 to 23.4°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.1 to 8.5, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 6.4 to 8.0 mg/L (≥75% 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, amphipods were collected from the holding container and placed in one or more transfer containers, then 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 and passed through an ultraviolet (UV) sterilizer to remove fine particles and microorganisms. 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® plastic beakers filled with approximately 20 mL of water. The depth of the test water in a representative chamber was 1.9 cm. The cups had a piece of Nitex® screen placed in each of the cup as substrate for the organisms. Test chambers were positioned in a temperature-controlled chamber to maintain a temperature of 23 ± 1°C. Test chambers were covered with plastic 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 200 mg a.i./L, the highest concentration tested, by mixing a calculated amount of PFOS into dilution water (Wildlife International, Ltd. UV sterilized well water). The stock solution for day 0 was mixed by stirring overnight and was sonicated for approximately 5 to 10 minutes the following day. For the day 2 solution, the stock solution was mixed by stirring overnight and was sonicated for 5 minutes the next

day. Both stock solutions appeared clear and colorless. Aliquots of the 200 mg a.i./L stock solution were proportionally diluted with well water to prepare 300 mL of test solution at nominal concentrations of 13, 25, 50 and 100 mg a.i./L. The solutions were mixed by inversion and all appeared clear and colorless. 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. All surviving amphipods 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 old test solutions were collected from each test chamber and pooled 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 Series 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 an Agilent 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 2.00 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 (40.0). 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 5.00, 50.0 and 250 mg a.i./L. The measured concentrations for the matrix fortification samples ranged from 98.4 to 114% 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 668 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 chambers 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 a composite sample of negative control water 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 amphipod. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4.5, 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 probability was used to calculate the 24-hour LC50 value and the probit analysis was used to calculate the 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 13, 25, 50, 100 and 200 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 100 to 105% of the nominal concentrations. Samples collected prior to renewal of the test solutions at 48 hours (old solutions), at renewal (new solutions) and at test termination had measured concentrations that ranged from 96.4 to 103%, 95 to 100% and 100 to 102% of the nominal concentrations, respectively. When measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations for this study were 13, 25, 51, 102 and 199 mg a.i./L, representing 100, 100, 102, 102 and 99.5% 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 each test chamber are presented in Table 2. Water temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations remained ≥8.1 mg/L (≥95% of saturation) throughout the test. Measurements of pH ranged from 8.1 to 8.6. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation 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. Amphipods in the negative control group appeared normal throughout the test, with the exception of two lethargic amphipods that resulted in mortality at test termination. This was well within an acceptable level as indicated in the guideline. Percent mortality at test termination in the 13, 25 and 51 mg a.i./L treatment groups was 40, 80, and 80%, respectively. There was 100% mortality in the 102 and 199 mg a.i/L PFOS treatment groups. The no-mortality concentration and the NOEC were both <13 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 amphipod, *Hyalella azteca*, was exposed for 96 hours under static-renewal conditions to five mean measured concentrations of PFOS ranging from 13 to 199 mg a.i./L. The 96-hour LC50 value was 15 mg a.i./L, with a 95% confidence interval of 4.5 to 24 mg a.i./L. The slope of the concentration-response curve was 2.4. The no-mortality concentration and the NOEC were both <13 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-253A-)	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	--		
13	2	0(new)	13.4	103	13	100
	8	48(new)	12.3	95.0		
	14	48(old)	13.5	103		
	20	96(old)	13.0	100		
25	3	0(new)	25.1	100	25	100
	9	48(new)	24.3	97.0		
	15	48(old)	24.9	99.6		
	21	96(old)	25.4	101		
50	4	0(new)	52.6	105	51	102
	10	48(new)	49.4	98.9		
	16	48(old)	50.2	100		
	22	96(old)	50.8	102		
100	5	0(new)	102	102	102	102
	11	48(new)	100	100		
	17	48(old)	103	103		
	23	96(old)	101	101		
200	6	0(new)	204	102	199	99.5
	12	48(new)	--[3]	--[3]		
	18	48(old)	193	96.4		
	24	96(old)	--[3]	--[3]		

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

[2] The limit of quantitation (LOQ) was 2.00 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 (40.0).

[3] Study level not sampled due to 100% mortality. Value not used in statistical calculations.

Table 2

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

Nominal 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	Temp.[1] (°C)	DO[2] (mg/L)	pH	Temp.[1] (°C)	DO[2] (mg/L)	pH	Temp.[1] (°C)	DO[2] (mg/L)	pH
Negative Control	22.4	8.3	8.2	23.3	8.2	8.6	23.4	8.4	8.2	23.1	8.5	8.5
	22.5	--	--	23.4	--	--	23.7	--	--	23.3	--	--
13	22.2	8.4	8.2	23.4	8.1	8.6	23.4	8.4	8.2	23.4	8.4	8.5
	22.4	--	--	23.3	--	--	23.7	--	--	23.1	--	--
25	22.6	8.5	8.2	23.4	8.1	8.6	23.4	8.5	8.3	23.1	8.4	8.5
	22.7	--	--	23.3	--	--	23.5	--	--	23.1	--	--
50	22.7	8.5	8.2	23.0	8.2	8.6	23.4	8.4	8.2	23.3	8.3	8.5
	23.1	--	--	23.3	--	--	23.8	--	--	23.4	--	--
100	22.9	8.5	8.2	23.1	8.2	8.6	23.6	8.4	8.3	23.4[4]	8.3	8.5
	23.1	--	--	23.2	--	--	23.7	--	--	23.3[4]	--	--
200	23.2	8.4	8.1	23.2	8.1	8.6	23.7[3]	--	--	--	--	--
	23.4	--	--	23.3	--	--	23.7[3]	--	--	--	--	--

[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 23.5 to 23.8°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 when appropriate volume of solutions were available. 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 prior to observation in replicates D and J, due to 100% mortality, and discontinued.

[4] Manual temperature measurements were taken at 96 hours in replicates A and H due to 100% mortality.

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)	295	320
Hardness (mg/L as $CaCO_3$)	132	136
Alkalinity (mg/L as $CaCO_3$)	182	186

Table 4

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate.[1]	4.5 Hours		24 Hours		48 Hours (T)		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	10
	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	C	1	--	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	
	K	0	AN	0	AN	0	C	1	--	1	--	
	L	0	AN	0	AN	0	AN	0	AN	0	AN	
	M	0	AN	0	AN	0	AN	0	AN	0	AN	
	N	0	AN	0	AN	0	AN	0	AN	0	AN	
	O	0	AN	0	AN	0	AN	0	AN	0	AN	
	P	0	AN	0	AN	0	AN	0	AN	0	AN	
	Q	0	AN	0	AN	0	AN	0	AN	0	AN	
	R	0	AN	0	AN	0	AN	0	AN	0	AN	
	S	0	AN	0	AN	0	AN	0	AN	0	AN	
	T	0	AN	0	AN	0	AN	0	AN	0	AN	
Cumulative No. Dead		0		0		0		1		2		

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

Table 4 (Continued)

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	4.5 Hours		24 Hours		48 Hours (T)		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]	
13	A	0	AN	0	AN	0	AN	0	AN	0	AN	40
	B	0	AN	0	AN	0	AN	0	C	1	--	
	C	0	AN	0	AN	0	AN	0	AN	1	--	
	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	C	1	--	1	--	
	J	0	AN	0	AN	0	AN	0	AN	1	--	
Cumulative No. Dead		0		0		0		1		4		
25	A	0	AN	0	AN	0	AN	0	C	1	--	80
	B	0	AN	0	AN	0	C	1	--	1	--	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	0	C	1	--	
	E	0	AN	0	AN	0	AN	1	--	1	--	
	F	0	AN	0	AN	0	AN	0	AN	1	--	
	G	0	AN	0	AN	0	AN	0	C	1	--	
	H	0	AN	0	AN	0	AN	1	--	1	--	
	I	0	AN	0	AN	0	C	1	--	1	--	
	J	0	AN	0	AN	0	AN	0	AN	0	AN	
Cumulative No. Dead		0		0				4		8		

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

Table 4 (Continued)

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	4.5 Hours		24 Hours		48 Hours (T)		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]	
51	A	0	AN	0	AN	0	C	1	--	1	--	80
	B	0	AN	0	AN	1	--	1	--	1	--	
	C	0	AN	0	AN	0	AN	0	AN	0	AN	
	D	0	AN	0	AN	0	AN	1	--	1	--	
	E	0	AN	0	AN	0	AN	1	--	1	--	
	F	0	AN	0	AN	0	AN	1	--	1	--	
	G	0	AN	0	AN	0	AN	1	--	1	--	
	H	0	AN	0	AN	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	C	1	--	1	--	
	J	0	AN	0	AN	0	C	1	--	1	--	
Cumulative No. Dead		0		0		1		8		8		
102	A	0	AN	0	AN	0	C	1	--	1	--	100
	B	0	AN	0	AN	0	C	1	--	1	--	
	C	0	AN	0	AN	1	--	1	--	1	--	
	D	0	AN	0	AN	0	C	1	--	1	--	
	E	0	AN	0	C	1	--	1	--	1	--	
	F	0	AN	0	AN	0	C	1	--	1	--	
	G	0	AN	0	AN	1	--	1	--	1	--	
	H	0	AN	0	AN	0	C	1	--	1	--	
	I	0	AN	0	AN	1	--	1	--	1	--	
	J	0	AN	0	AN	1	--	1	--	1	--	
Cumulative No. Dead		0		0		5		10		10		

[1] A single amphipod was exposed in each replicate.
[2] Number of dead amphipods at time of observation.
[3] Observations: AN = appear normal; C = lethargy.
[4] T = transferred to new test solution.

Table 4 (Continued)

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	4.5 Hours		24 Hours		48 Hours (T)		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]	
199	A	0	AN	1	--	1	--	1	--	1	--	100
	B	0	AN	1	--	1	--	1	--	1	--	
	C	0	AN	1	--	1	--	1	--	1	--	
	D	0	AN	0	AN	1	--	1	--	1	--	
	E	0	AN	1	--	1	--	1	--	1	--	
	F	0	AN	1	--	1	--	1	--	1	--	
	G	0	AN	1	--	1	--	1	--	1	--	
	H	0	AN	1	--	1	--	1	--	1	--	
	I	0	AN	1	--	1	--	1	--	1	--	
	J	0	AN	0	AN	1	--	1	--	1	--	
Cumulative No. Dead		0		8		10		10		10		

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

Table 5

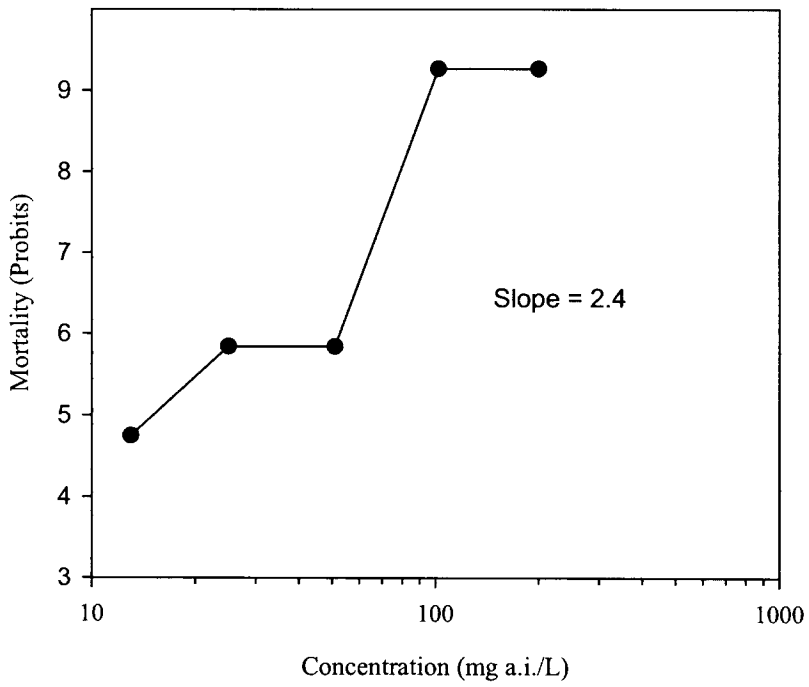
LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	161	>102[1]	Binomial Probability
48 Hours	93	70 - 125	Probit Analysis
72 Hours	29	20 - 40	Probit Analysis
96 Hours	15	4.5 – 24	Probit Analysis

[1] At a confidence level of 95% the binomial test shows that the LC50 is above 102 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)	290 (N = 4)	285 - 295
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)	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 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:	Agilent 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% MeOH : 20% H_20 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 10.0 and 1.00 mg a.i./mL stock solutions were used to prepare concurrent matrix fortification samples (QC) for this study. The 0.100 mg a.i./mL 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-253A-2, an exposure sample prepared at a nominal concentration of 13 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-253A-2 are summarized below:

Peak area = 1460100
Constant Coefficient = -3901.34
Linear Coefficient = 4406500
Quadratic Coefficient = -80517.7
Dilution Factor ($V_{final}/V_{initial}$): = 40.0

Appendix 3.4 (Continued)

Example Calculations for a Representative Sample

$$PFOS = 40.0 \bullet \frac{-4406500 + \sqrt{(4406500)^2 - [(4 \bullet (-80517.7)) \bullet (-3901.34 - 1460100)]}}{2 \bullet (-80517.7)}$$

PFOS = 40.0 • 0.33425 mg a.i./L

PFOS = 13.4 mg a.i./L

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

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

$$= \frac{13.4 \text{ mg/L}}{13.0 \text{ mg/L}} \text{ X } 100$$

= 103%

Appendix 3.5

Quality Control Samples of PFOS in Freshwater

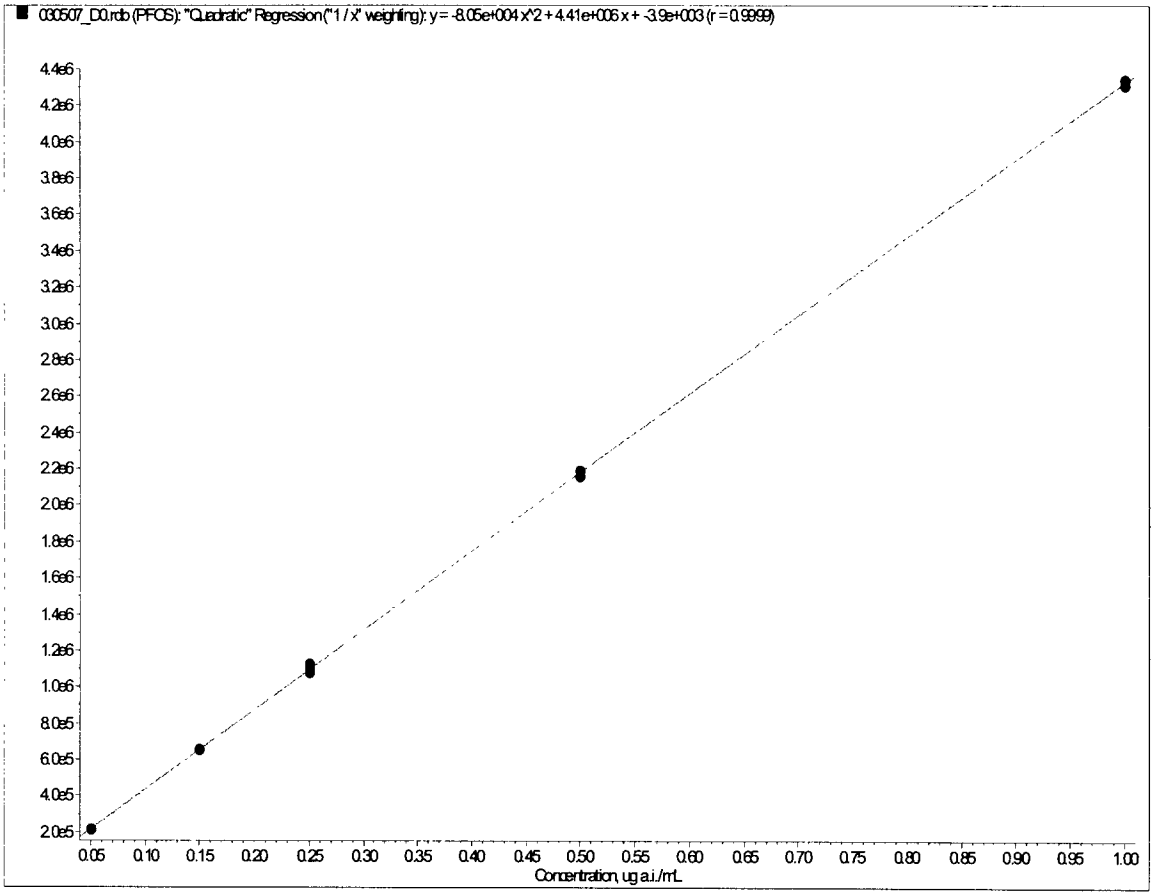
Sample Number (454A-253A-)	Sampling Time (Hours)	Concentration (mg a.i./L)		Percent Recovery [1]
		Fortified	Measured [1,2]	
MAB-1	0	0.0	< LOQ	--
MAB-2	48	0.0	< LOQ	--
MAB-3	96	0.0	< LOQ	--
MAS-1	0	5.00	5.51	110
MAS-2	0	50.0	51.5	103
MAS-3	0	250	251	100
MAS-4	48	5.00	5.71	114
MAS-5	48	50.0	52.4	105
MAS-6	48	250	246	98.4
MAS-7	96	5.00	4.98	99.5
MAS-8	96	50.0	49.5	99.0
MAS-9	96	250	246	98.5
				X=103 S.D.=5.62 C.V.=5.46%

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

[2] The limit of quantitation (LOQ) was 2.00 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 (40.0).

Appendix 3.6

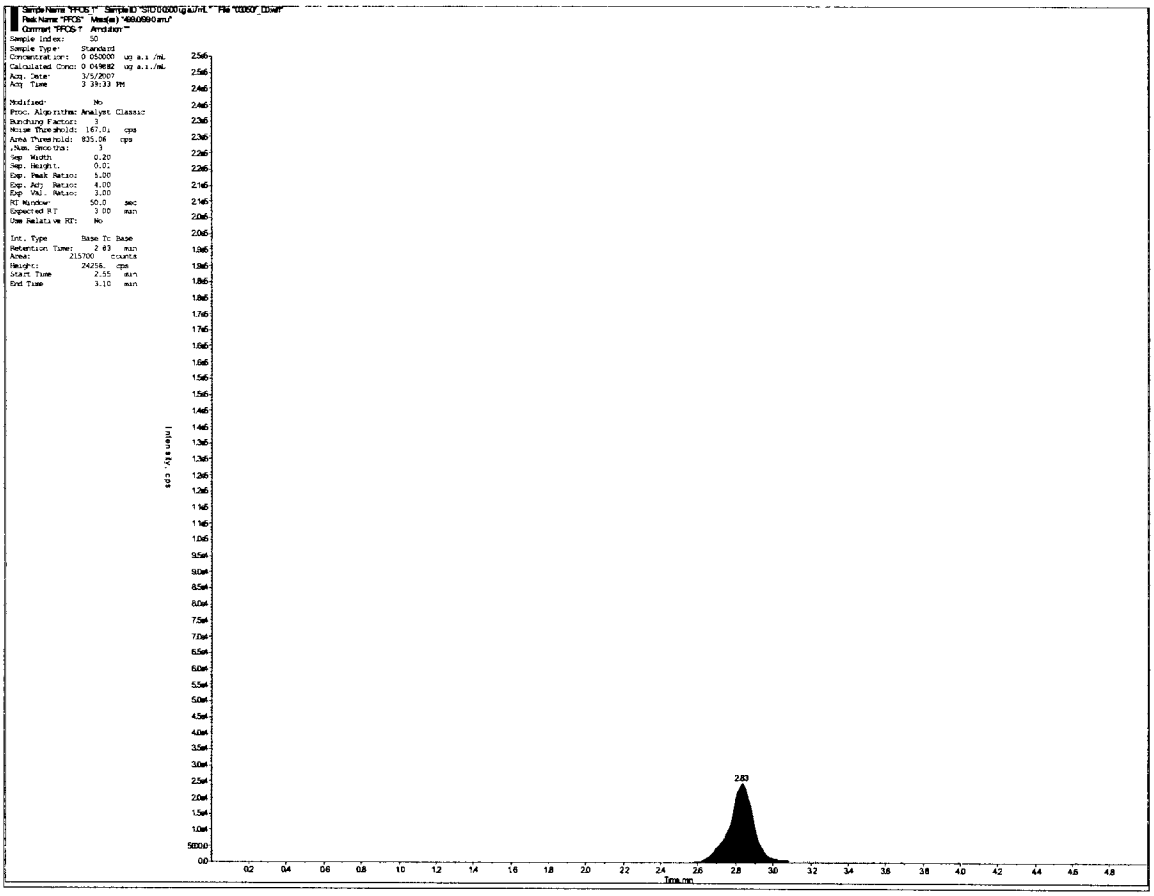
Representative Calibration Curve for PFOS



Linear coefficient=4406500; constant coefficient=-3901.34; quadratic coefficient= -80517.7; r=0.9999

Appendix 3.7

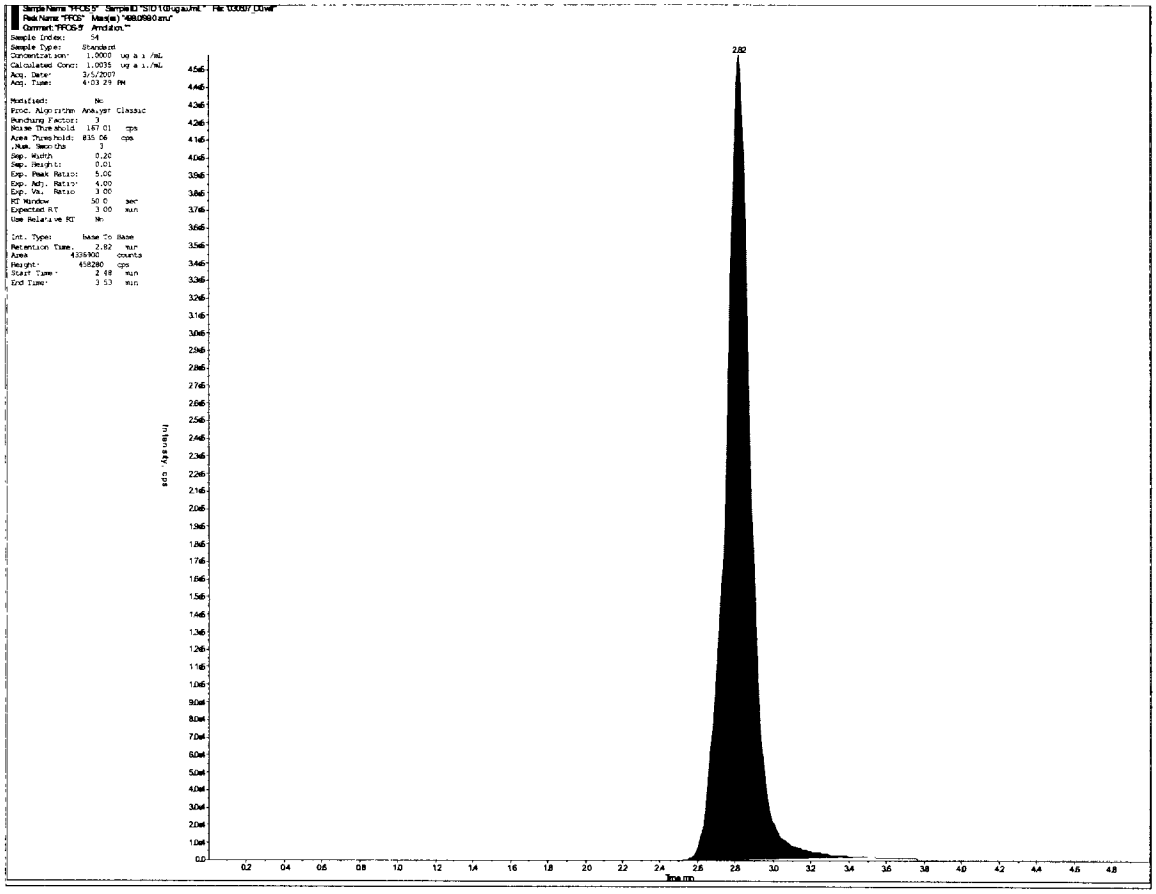
Representative Chromatogram of a Low-level PFOS Calibration Standard



Nominal concentration: 0.0500 mg a.i./L

Appendix 3.8

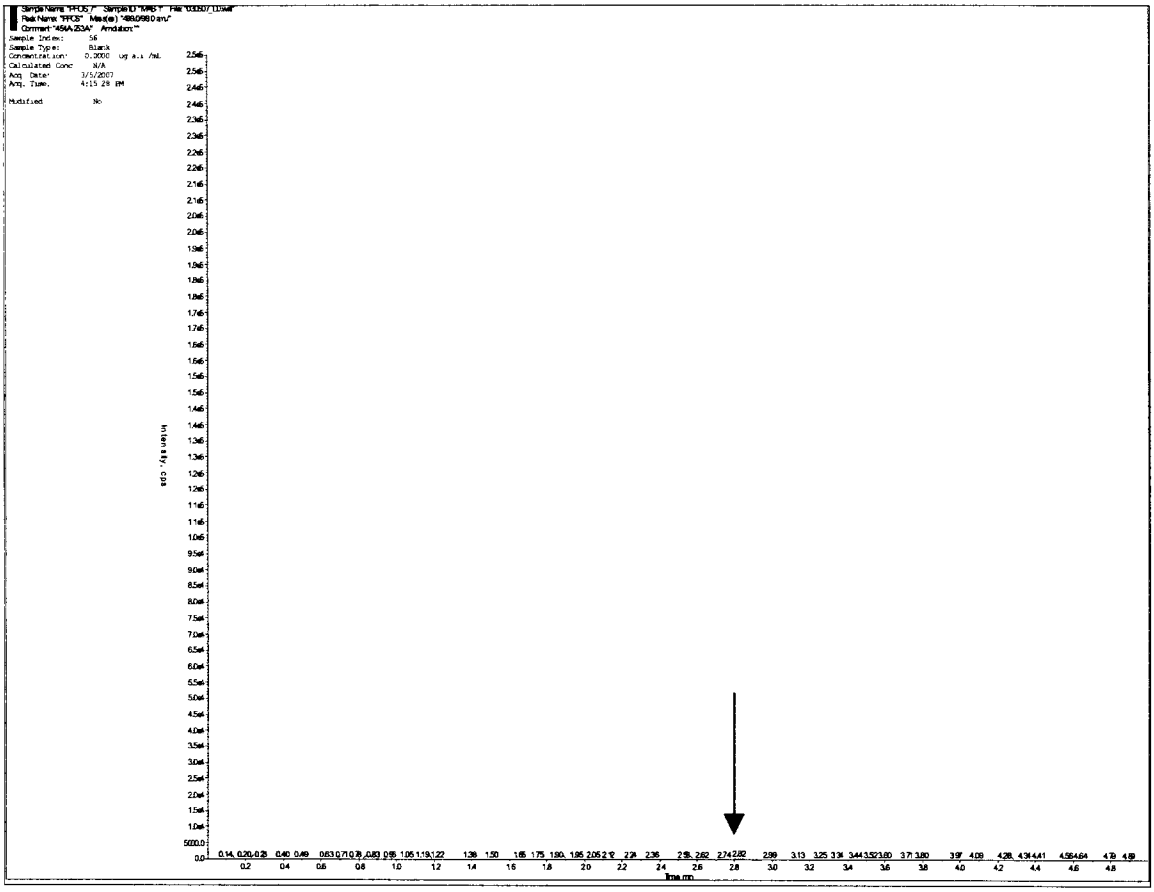
Representative Chromatogram of a High-level PFOS Calibration Standard



Nominal concentration: 1.00 mg a.i./L

Appendix 3.9

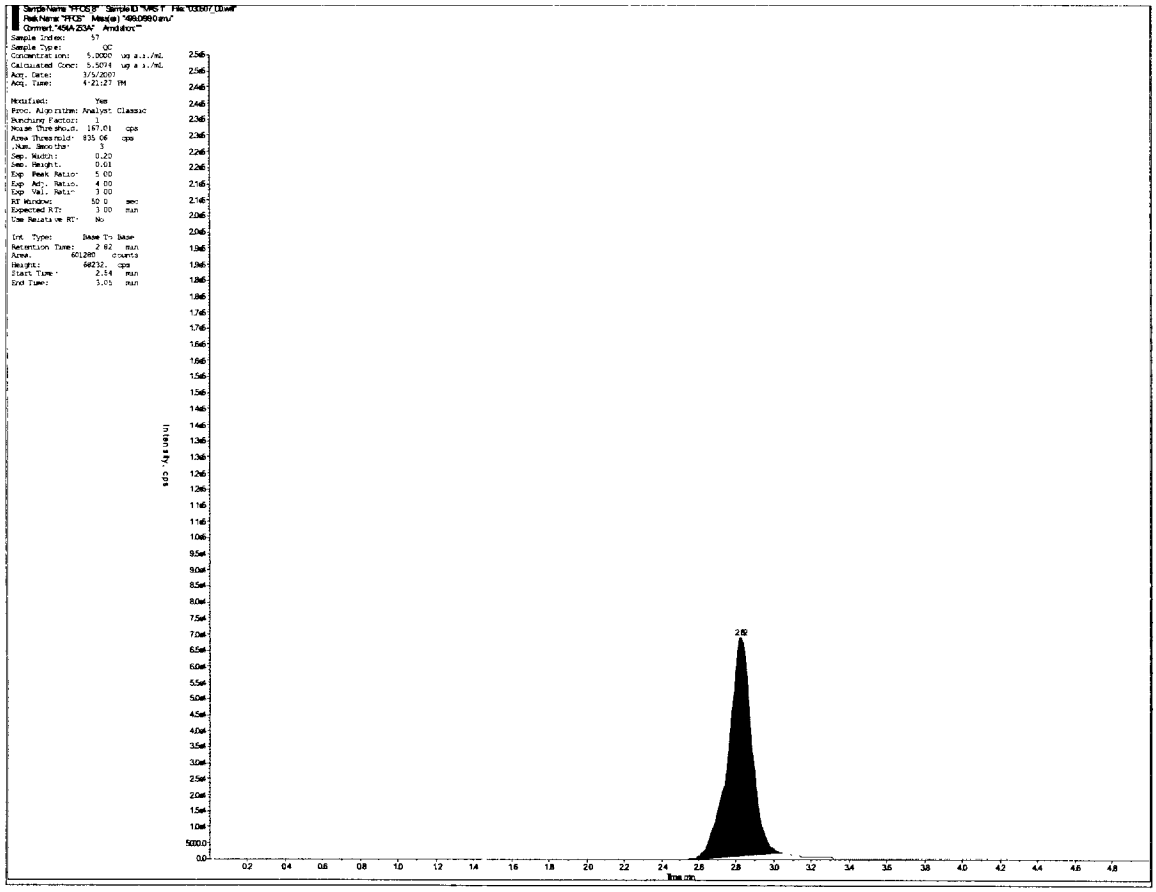
Representative Chromatogram of a Matrix Blank Sample



Sample number 454A-253A-MAB-1. Dilution factor = 40.0X. The arrow indicates the retention time of PFOS.

Appendix 3.10

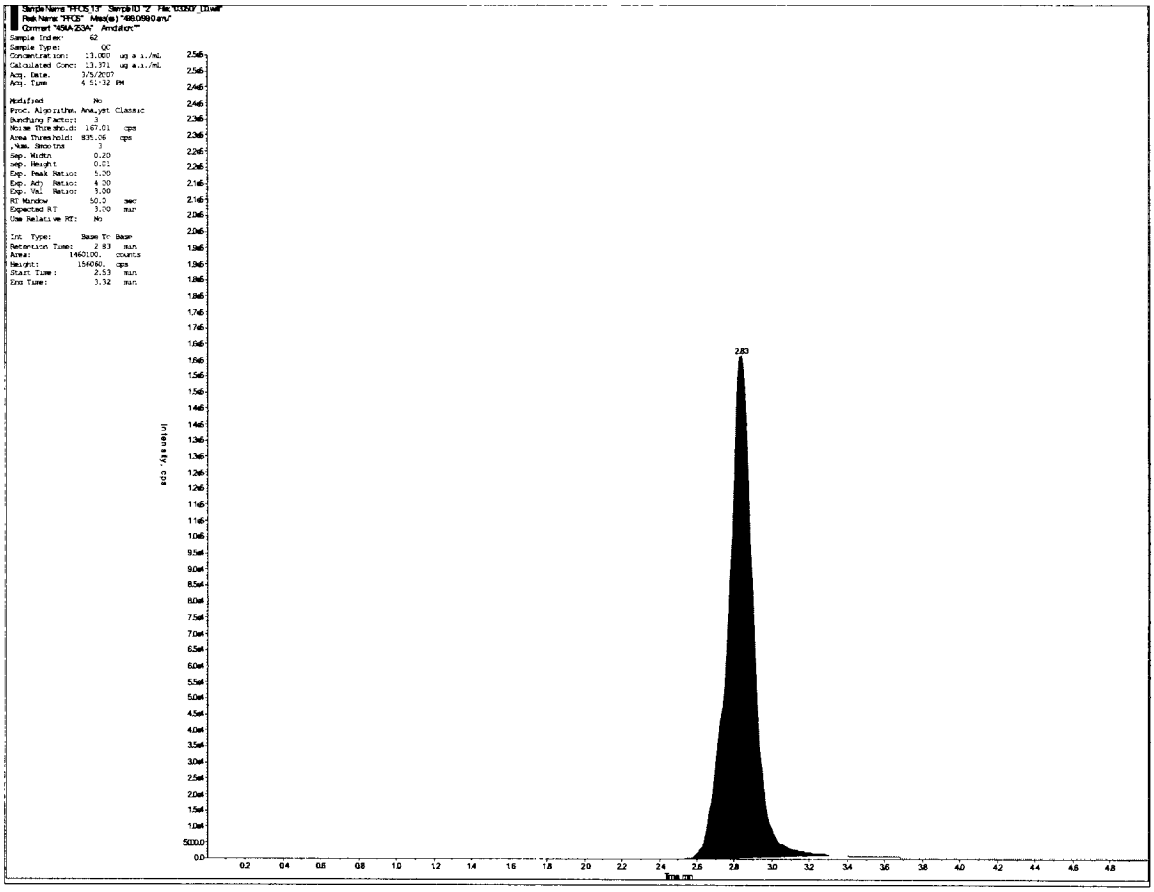
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 454A-253A-MAS-1, nominal concentration 5.00 mg/L. Dilution factor = 40.0X.

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 454A-253A-2, Day 0, nominal concentration 13 mg a.i./L. Dilution factor = 40.0X.

Appendix 4

Changes to Protocol

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

1. The test chambers had a piece of Nitex® screen placed in each of the beakers as substrate.

2. The temperature was measured 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.

3. The temperature was monitored 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.

4. Dissolved oxygen and pH were measured 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.

5. A tray of clean water was placed in the environmental chamber to maintain the moisture content of the air in the environmental chamber and thus decrease the rate of evaporative loss of water from the test solutions in the test chambers.

6. Test organisms were obtained from Environmental Consulting and Testing, Superior, WI.

7. The Environmental Laboratory Project Number assigned by the Sponsor for this study was E07-082.

8. Twenty replicate test chambers were maintained in the negative control group while ten replicate test chambers were maintained in each treatment group. A single organism was placed in each test chamber for a total of twenty organisms per negative control group, and ten organisms per each treatment group.

9. Dilution water was passed through a UV sterilizer prior to use. This had no adverse impact on the study results.

10. The test solution level in each chamber was not examined and brought up to the 20 mL mark daily with reverse osmosis water. 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