PFOA: A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH *Chironomus tentans*

AMENDED FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-250
3M ENVIRONMENTAL LABORATORY PROJECT NUMBER: E07-0081

ASTM Standard E729-96

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STUDY INITIATION DATE: February 7, 2007

STUDY COMPLETION DATE: March 2, 2007

AMENDED FINAL REPORT DATE: March 8, 2007

SUBMITTED TO:

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AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR 3M Corporation

TITLE PFOA A 96-Hour Static-Renewal Acute Toxicity Test with *Chironomus tentans*

WILDLIFE INTERNATIONAL, LTD PROJECT NUMBER 454A-250

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AMENDED REPORT DATE March 8, 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

The test substance was characterized in compliance with Good Laboratory Practice Standards prior to the start of the test However, test solutions were prepared and used, and the test was carried out, after the test substance expiration date of October 31, 2006 while the Certificate of Analysis was being updated

STUDY DIRECTOR

Tui Minderhout

Tui Minderhout, Ph D
Senior Biologist

3/8/2007
Date

SPONSOR APPROVAL

Sponsor Representative

3/16/2007
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 (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 12, 2007	February 12, 2007	February 15, 2007
Test Substance Preparation	February 12, 2007	February 12, 2007	February 21, 2007
Matrix Fortification	February 16, 2007	February 16, 2007	February 21, 2007
Water Chemistry	February 16, 2007	February 16, 2007	February 21, 2007
Analytical Data and Draft Report	February 22 – 23, 2007	February 23, 2007	March 1, 2007
Biological Data and Draft Report	February 22 - 23, 2007	February 23, 2007	February 27, 2007
Final Report	March 2, 2007	March 2, 2007	March 2, 2007
Amended Final Report	March 8, 2007	March 8, 2007	March 8, 2007

All inspections were study-based unless otherwise noted.

James H. Coleman 3-8-07

James H. Coleman Date
Quality Assurance Representative

AMENDED

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOA: A 96-Hour Static-Renewal Acute Toxicity Test with *Chironomus tentans*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-250

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AMENDED

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SUMMARY

SPONSOR: 3M Corporation

TITLE: PFOA: A 96-Hour Static-Renewal Acute Toxicity Test with *Chironomus tentans*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-250

3M ENVIRONMENTAL LABORATORY PROJECT NUMBER: E07-0081

TEST DATES:	Experimental Start:	February 12, 2007
	Biological Termination:	February 16, 2007
	Experimental Termination:	February 16, 2007

LENGTH OF EXPOSURE: 96 Hours

TEST ORGANISMS: Freshwater Midge (*Chironomus tentans*)

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
Superior, WI 54880

AGE OF TEST ORGANISMS: 2nd to 3rd instar larvae (approximately 10-day old) at test start

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	< LOQ
63 mg a.i./L	74 mg a.i./L
125 mg a.i./L	153 mg a.i./L
250 mg a.i./L	277 mg a.i./L
500 mg a.i./L	579 mg a.i./L
1000 mg a.i./L	1090 mg a.i./L

RESULTS: Based on mean measured concentrations:

96-Hour LC50:	>1090 mg a.i./L
No-Mortality Concentration:	277 mg a.i./L
No-Observed-Effect Concentration:	277 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. The in-life phase of the definitive toxicity test was conducted from February 12 to 16, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454A-250 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the acute effects of perfluorooctanoate, ammonium salt (PFOA) on the freshwater midge, *Chironomus tentans*, during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Midge larvae 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 midges per concentration. 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 63, 125, 250, 500 and 1000 mg 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 after renewal at 48 hours, and at the end of the test.

Midge larvae were impartially assigned to test chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 2.5, 25, 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/immobility 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, "PFOA: A 96-Hour Static-Renewal Acute Toxicity Test with *Chironomus tentans*". 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 Decmber 27, 2006. It was assigned Wildlife International, Ltd. identification number 7864 upon receipt and was stored under frozen conditions. The test substance, a solid, was identified as: FC-143; PFOA; Lot number 332; CAS number 3825-26-1. The test substance contained 95% active ingredient and had an expiration date of February 27, 2017 (Appendix 1).

Test Organism

The midge, *Chironomus tentans*, was selected as the test species for this study. Midges 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. Midge larvae used in the test were second- to third-instar larvae (approximately 10 days old) at test initiation. Midge larvae were obtained from Environmental Consulting and Testing (ECT), Superior, Wisconsin and were 7 days old when received. During the 3-day period prior to the test, the organisms were held in an aquarium with sand and overlying water from the same source and at approximately the same temperature as that used in the test. Midges were fed a 56 g/L suspension of flake food during the holding period and a 4 g/L suspension of Tetramin® flake food on days 0 and 2 of the test.

During the 3-day acclimation period immediately preceding the test, water temperatures in the holding aquarium ranged from 22.0 to 22.8°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.1 to 8.4, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 7.2 to 8.6 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, midge larvae were collected from the holding aquarium 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 holding 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 2.

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 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 3.

Test Apparatus

Test chambers were 30-mL Nalgene® plastic beakers filled with approximately 20 mL of water. The cups had approximately one-eighth of a teaspoon of sand on the bottom of the cup to provide a monolayer of burrowing substrate for the midges. 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 1000 mg a.i./L, the highest concentration tested, by mixing a calculated amount of PFOA into dilution water. The stock solution was sonicated for five minutes and mixed by inversion, and appeared clear and colorless. Aliquots of the 1000 mg a.i./L stock solution were proportionally diluted with well water to prepare 300 mL of test solutions at nominal concentrations of 63, 125, 250 and 500 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 (95%). Test solutions were prepared on day 0 and day 2. All surviving

midge larvae were transferred from old to new solutions at approximately 48 hours. At test initiation and termination, all solutions appeared clear and colorless in the test chambers.

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 PFOA 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 PFOA 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 PFOA is provided in Appendix 4.1 and typical instrumental parameters are summarized in Appendix 4.2.

Calibration standards of PFOA, ranging in concentration from 0.100 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 PFOA in acetonitile (Appendix 4.3). 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 PFOA 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 4.4.

The method limit of quantitation (LOQ) for these analyses was set at 10.0 mg a.i./L, calculated as the product of the lowest calibration standard (0.100 µg a.i./mL) and the dilution factor of the matrix blank samples (100). Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 4.5).

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 PFOA concentrations of 25.0, 250 and 1200 mg a.i./L. The measured concentrations for the matrix fortification samples ranged from 97.0 to 107% of nominal concentrations (Appendix 4.5)

A representative calibration curve is presented in Appendix 4.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 4.7 and 4.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 4.9 and a representative chromatogram of a matrix fortification sample is presented in Appendix 4.10. A representative chromatogram of a test sample is presented in Appendix 4.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the holding 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 776 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 of each treatment and control group at the beginning of the test, prior to and after the 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 the batches of new test solution at 0 and 48 hours or were measured in composite samples of old solutions at 48 and 96 hours. 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 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 was defined as the lack of visible movement in the midge. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 2.5, 25, 48, 72 and 96 hours after test initiation.

Statistical Analyses

Less than 50% mortality in any of the PFOA treatment groups was observed during the test, which precluded the statistical calculation of LC50 values. Therefore, the 24-, 48-, 72- and 96-hour LC50 values were estimated to be greater than the highest concentration tested. The no-mortality/immobility concentration and the no-observed-effect concentration (NOEC) were determined by visual interpretation of the mortality, immobility and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations selected for use in this study were 63, 125, 250, 500 and 1000 mg a.i./L. Results of analyses to measure concentrations of PFOA 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 102 to 106% of the nominal concentrations. Samples collected prior to and after renewal of the test solutions at 48 hours had measured concentrations that ranged from 120 to 141% and 102 to 104%, respectively, of the nominal concentrations. Samples collected at test termination had measured concentrations that ranged from 107 to 160% of the nominal concentrations.

The concentration of the solutions collected after 48 and 96 hours were slightly higher than those of newly prepared solutions due in part to the evaporative loss of water from the tests solutions even though the test chambers were covered with plastic. The high surface area in comparison to the small volume of the solution and the position of the fan that circulated the air in the environmental chamber might have contributed to the evaporation rate. When measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations for this study were 74, 153, 277, 579 and 1090 mg a.i./L, representing 118, 123, 111, 116 and 109% of nominal concentrations, respectively. The mean measured concentrations indicated no reduction in test concentrations in the test system during the study. 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 ≥7.6 mg/L (≥89% of saturation) throughout the test. Measurements of pH ranged from 7.9 to 8.5. 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. A 10% mortality rate was observed in both the negative control group and in the 74 mg a.i./L

treatment group after 72 and 96 hours of exposure, respectively. Since the mortality in the 74 mg a.i./L treatment group was comparable to the negative control, and was not dose-responsive, it was not considered to be treatment-related. No mortalities or signs of toxicity were observed among midges in the 153 and 277 mg a.i./L treatment groups during the test. Percent mortality at test termination in the 579 and 1090 mg a.i./L treatment groups was 30 and 40%, respectively. All surviving midges in the PFOA treatment groups appeared normal throughout the test. The no-mortality concentration and the NOEC were both 277 mg a.i./L. LC50 values at 24, 48, 72 and 96 hours were estimated to be >1090 mg a.i./L, the highest concentration tested (Table 5).

CONCLUSIONS

The midge, *Chironomus tentans*, was exposed for 96 hours under static-renewal conditions to five mean measured concentrations of PFOA ranging from 74 to 1090 mg a.i./L. The 96-hour LC50 value was >1090 mg a.i./L, the highest concentration tested. Based on the mortality seen in the 579 and 1090 mg a.i./L treatment groups, the 96-hour no-mortality concentration and the NOEC were both 277 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.

Table 1

Measured Concentrations of PFOA in Freshwater Samples

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-250-)	Sampling Time (Hours)	Measured Concentration PFOA (mg a.i./L) [1]	Percent of Nominal [1]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
0.00	1	0(new)	< LOQ[2]	--	< LOQ	--
(Negative Control)	7	48(new)	< LOQ	--		
	13	48(old)	< LOQ	--		
	19	96(old)	< LOQ	--		
63	2	0(new)	65.4	104	74	117
	8	48(new)	65.1	103		
	14	48(old)	75.5	120		
	20	96(old)	91.2	145		
125	3	0(new)	130	104	153	122
	9	48(new)	129	103		
	15	48(old)	154	123		
	21	96(old)	200	160		
250	4	0(new)	259	104	277	111
	10	48(new)	259	104		
	16	48(old)	302	121		
	22	96(old)	289	116		
500	5	0(new)	532	106	579	116
	11	48(new)	511	102		
	17	48(old)	707	141		
	23	96(old)	566	113		
1000	6	0(new)	1020	102	1090	109
	12	48(new)	1020	102		
	18	48(old)	1260	126		
	24	96(old)	1070	107		

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

[2] The limit of quantitation (LOQ) was 10.0 mg a.i./L calculated as the product of the lowest calibration standard (0.100 µg a.i./mL) and the dilution factor of the matrix blanks (100).

Table 2

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

Mean Measured Concentration (mg a.i./L)	0 Hour			48 Hours (Prior to Renewal)			48 Hours (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	22.5	8.7	8.2	22.4	7.9	8.5	22.4	8.7	8.3	22.4	8.0	8.5
	22.3	--	--	22.0	--	--	22.2	--	--	22.6	--	--
74	22.7	8.7	8.2	22.0	8.0	8.5	22.6	8.6	8.3	22.1	8.0	8.5
	23.0	--	--	22.0	--	--	22.2	--	--	22.1	--	--
153	22.5	8.7	8.1	21.9	8.0	8.4	22.4	8.7	8.3	22.1	8.0	8.5
	22.5	--	--	21.8	--	--	22.1	--	--	22.5	--	--
277	22.4	8.7	8.1	21.7	8.1	8.4	22.4	8.7	8.2	22.8	8.0	8.4
	22.5	--	--	22.4	--	--	22.0	--	--	22.7	--	--
579	22.5	8.7	8.0	22.1	8.1	8.3	22.3	8.7	8.2	22.1	7.6	8.4
	22.5	--	--	22.8	--	--	22.5	--	--	22.8	--	--
1090	22.3	8.7	7.9	22.2	8.0	8.1	22.7	8.7	8.0	22.6	7.7	8.3
	23.0	--	--	22.9	--	--	22.3	--	--	22.8	--	--

[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 to 24°C.

[2] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C in freshwater.

Table 3

Specific Conductance, Hardness and Alkalinity Measured in Dilution Water at Test Initiation and Termination

Parameter	Day 0	Day 4
Specific Conductance (µmhos/cm)	290	310
Hardness (mg/L as $CaCO_3$)	132	136
Alkalinity (mg/L as $CaCO_3$)	180	182

Table 4

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	2.5 Hours		25 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	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	D	1	---	1	---	
	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	
74	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	D	1	---	
	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, D = pale.
[4] T = transferred to new test solution.

Table 4 (Continued)

Cumulative Mortality and Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	2.5 Hours		25 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]	
153	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	
277	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 Clinical Observations

Mean Measured Concentration (mg a.i./L)	Replicate[1]	2.5 Hours		25 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]	
579	A	0	AN	0	AN	0	AN	0	AN	0	AN	30
	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	1	---	
	E	0	AN	0	AN	0	AN	0	AN	0	AN	
	F	0	AN	0	AN	0	AN	0	AN	0	AN	
	G	1	---	1	---	1	---	1	---	1	---	
	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	1	---	1	---	1	---	1	---	
1090	A	0	AN	0	AN	0	D	1	---	1	---	40
	B	0	AN	0	AN	0	AN	0	AN	0	AN	
	C	0	AN	0	AN	0	AN	0	D	1	---	
	D	0	AN	0	AN	0	AN	0	AN	0	AN	
	E	0	AN	1	---	1	---	1	---	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	0	AN	0	AN	0	AN	
	I	0	AN	0	AN	0	AN	0	AN	0	AN	
	J	0	AN	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.
[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	> 1090	--[1]	NA[2]
48 Hours	> 1090	--[1]	NA[2]
72 Hours	> 1090	--[1]	NA[2]
96 Hours	> 1090	--[1]	NA[2]

[1] 95% confidence limits could not be calculated with the mortality/immobility data obtained.
[2] NA = not applicable; <50% mortality precluded statistical calculation of an LC50 value.

Appendix 1

Certificate of Analysis

INTERIM CERTIFICATE OF ANALYSIS

Revision 3

Centre Analytical Laboratories COA Reference #: 023-034
3M Product: PFOA, Primary Standard
Test Control Reference #: TCR-99030-30
Purity: 95.0%

Test Name	Specifications	Result
Purity[1]		**95.0%**
Appearance	White, crystalline solid	Conforms
Identification NMR		Positive
Metals (ICP/MS) 1. Calcium 2. Magnesium 3. Sodium 4. Potassium 5. Nickel 6. Iron 7. Manganese		 1. 0.001 wt./wt.% 2. <0.001 wt./wt.% 3. 0.001 wt./wt.% 4. <0.001 wt./wt.% 5. <0.001 wt./wt.% 6. <0.001 wt./wt.% 7. <0.001 wt./wt.%
Total % Impurity (NMR)		0.36 wt./wt.%
Total % Impurity (LC/MS)		4.68wt./wt.%
Total % Impurity (GC/MS)		None Quantified.%
Residual Solvents (TGA)		None Detected
Purity by DSC		99.8%
Inorganic Anions (IC) 1. Chloride 2. Fluoride 3. Bromide 4. Nitrate 5. Nitrite 6. Phosphate 7. Sulfate		 1. <0.015 wt./wt.% 2. <0.005 wt./wt.% 3. <0.040 wt./wt.% 4. <0.009 wt./wt.% 5. <0.006 wt./wt.% 6. <0.006 wt./wt.% 7. <0.040 wt./wt.%
Organic Acids[2] (IC) 1. TFA 2. PFPA 3. HFBA 4. NFPA		 1. <0.1 wt./wt.% 2. <0.1 wt./wt.% 3. <0.1 wt./wt.% 4. <0.25 wt./wt.%
Elemental Analysis[3]: 1. Carbon 2. Hydrogen 3. Nitrogen 4. Sulfur 5. Fluorine	 1. Theoretical Value = 22.3% 2. Theoretical Value = 0.935% 3. Theoretical Value = 3.25% 4. Theoretical Value = 0% 5. Theoretical Value = 66.1%	 1. 18.9 wt./wt.% 2. 1.27 wt./wt.% 3. 3.76 wt./wt.% 4. 4.38 wt./wt.% 5. 62.1 wt./wt.%
Ammonium Analysis[3] Ion Selective Electrode	Theoretical Value = 4.18%	2.94 wt./wt. %

AMENDED

INTERIM CERTIFICATE OF ANALYSIS

Revision 3

Centre Analytical Laboratories COA Reference #: 023-034
3M Product: PFOA, Primary Standard
Test Control Reference #: TCR-99030-30

Date of Last Analysis: 2/27/07

Expiration Date: 2/27/17

Storage Conditions: < -10 °C

Re-assessment Date: 2/27/17

[1]Purity = 100% - (total metal impurities, 0.002% + Total NMR impurities, 0.36 + Total LC/MS impurities, 4.68 %)

Total impurity from all tests = 5.042%
Purity = 100% - 5.042% = 95.0%

[2] TFA Trifluoroacetic acid
HFBA Heptafluorobutyric acid
NFPA Nonafluoropentanoic acid
PFPA Pentafluoropropanoic acid

[3]Theoretical value calculations based on the empirical formula, $C_8F_{15}O_2^{(-)}NH_4^{(+)}$ (MW=431.1)

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

LC/MS Purity Profile:

Peak #	Retention Time (min)	Mass(s)	Identity	Area	% Area
1	12.144	269.0	C6	205859	0.87
2	13.533	331, 319	F_{13}/C7	903329	3.81
3	14.238	369	PFOA	22630600	-
Total	-	-	-	23739788	4.68

Prepared By: _____ 3/2/07
Charles Simons Date
Exygen Research

Reviewed By: _____ 2-Mar-07
Kevin Lloyd Date
Exygen Research

AMENDED

Appendix 2

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)	293 (N = 4)	285 – 300
Hardness (mg/L as $CaCO_3$)	136 (N = 4)	132 – 140
Alkalinity (mg/L as $CaCO_3$)	183 (N = 4)	182 – 184
pH	8.0 (N = 4)	7.9 – 8.1

Appendix 3

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 3 (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 4

The Analysis of PFOA in Freshwater

Appendix 4.1

Analytical Method Flowchart for the Processing of PFOA in Freshwater

METHOD OUTLINE FOR THE ANALYSIS OF PFOA 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 4.2

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:	5.00 µL
PFOA RETENTION TIME:	Approximately 2.6 minutes
PFOA MONITORED MASS:	413 → 369 amu

Appendix 4.3

Analytical Stocks and Standards Preparation

A calibration standard stock solution of PFOA was prepared by weighing 0.1053 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 acetonitile. This primary stock solution contained 1.00 mg a.i./mL of PFOA. A secondary stock of PFOA in acetonitile (0.100 mg a.i./mL) was prepared from the primary stock by volumetric dilution. 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	0.100	100	0.100
0.100	0.250	100	0.250
0.100	0.500	100	0.500
0.100	0.750	100	0.750
0.100	1.00	100	1.00

A fortification standard stock solution of PFOA was prepared by weighing 1.0526 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 PFOA and was used to prepare concurrent matrix fortification (QC) samples.

Appendix 4.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 454A-250-2, an exposure sample prepared at a nominal concentration of 63 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 PFOA 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 PFOA in mg a.i./L

a = quadratic coefficient

b = linear coefficient

c =constant coefficient (y_intercept)

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

PFOA (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 PFOA in Sample Number 454A-250-2 are summarized below:

Peak area = 15456000
Constant Coefficient = 32311.9
Linear Coefficient = 26602900
Quadratic Coefficient = -4615070
Dilution Factor ($V_{final}/V_{initial}$): = 100

Appendix 4.4 (Continued)

Example Calculations for a Representative Sample

$$PFOA = 100 \bullet \frac{-26602900 + \sqrt{(-26602900)^2 - [(4 \bullet (-4615070)) \bullet (32311.9 - 15456000)]}}{2 \bullet (-4615070)}$$

PFOA = 100 • 0.654 mg a.i./L

PFOA = 65.4 mg a.i./L

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

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

$$= \frac{65.4 \text{ mg/L}}{63.0 \text{ mg/L}} \text{ X } 100$$

= 104%

Appendix 4.5

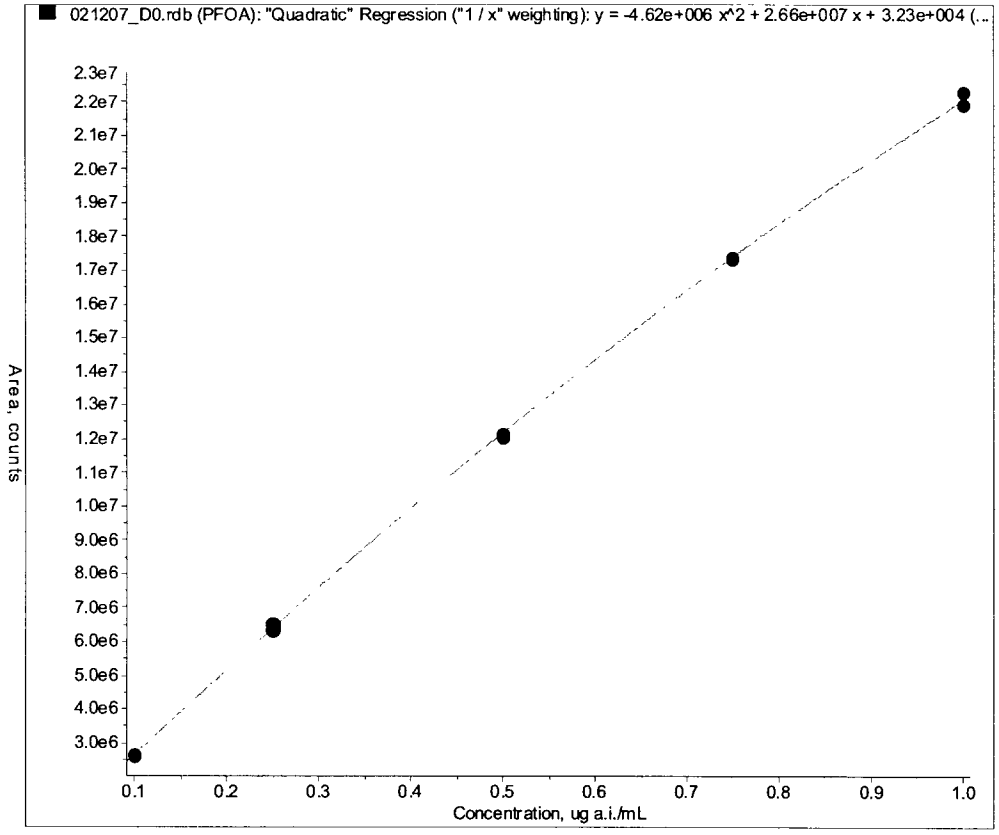
Quality Control Samples of PFOA in Freshwater

Sample Number (454A-250-)	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	25.0	26.9	107
MAS-2	0	250	258	103
MAS-3	0	1200	1190	99.1
MAS-4	48	25.0	25.3	101
MAS-5	48	250	254	102
MAS-6	48	1200	1200	99.7
MAS-7	96	25.0	25.9	104
MAS-8	96	250	256	102
MAS-9	96	1200	1160	97.0
				Mean = 102 S.D. = 2.93 C.V. = 2.87%

[1] Results generated using Analyst version 1.4.1 software. Manual calculations may vary.
[2] The limit of quantitation (LOQ) was 10.0 mg a.i./L calculated as the product of the lowest calibration standard (0.100 µg a.i./mL) and the dilution factor of the matrix blanks (100).

Appendix 4.6

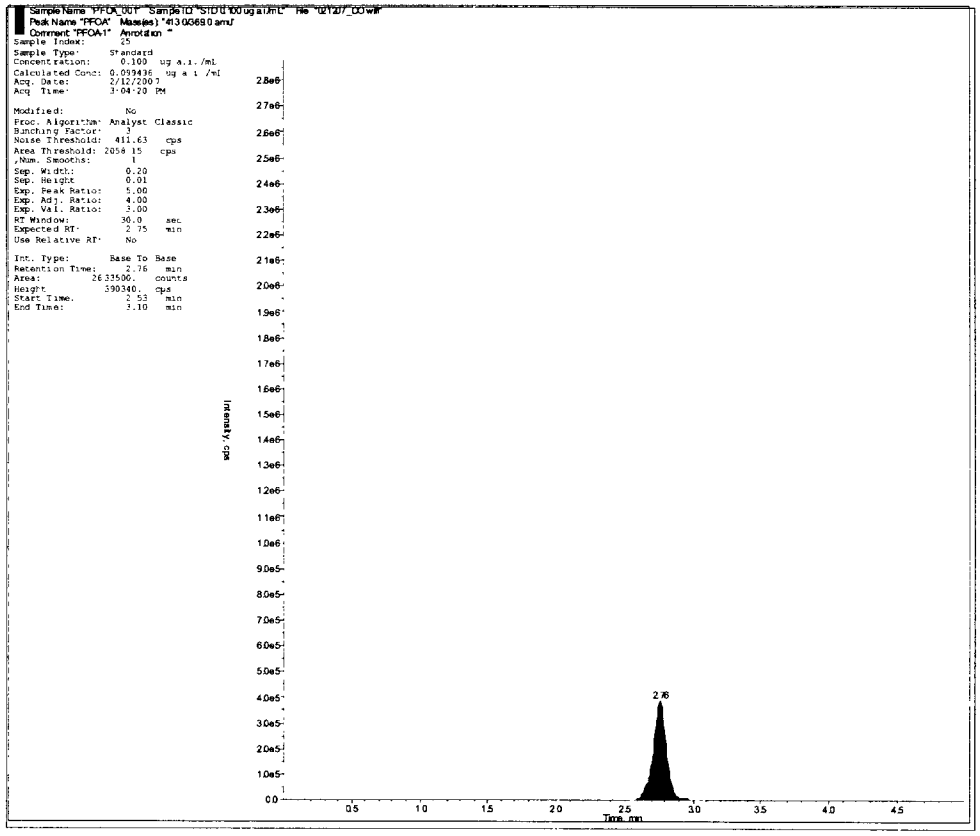
Representative Calibration Curve for PFOA



Linear coefficient=26602900; constant coefficient=32311.9; quadratic coefficient=-4615070; r=0.9999

Appendix 4.7

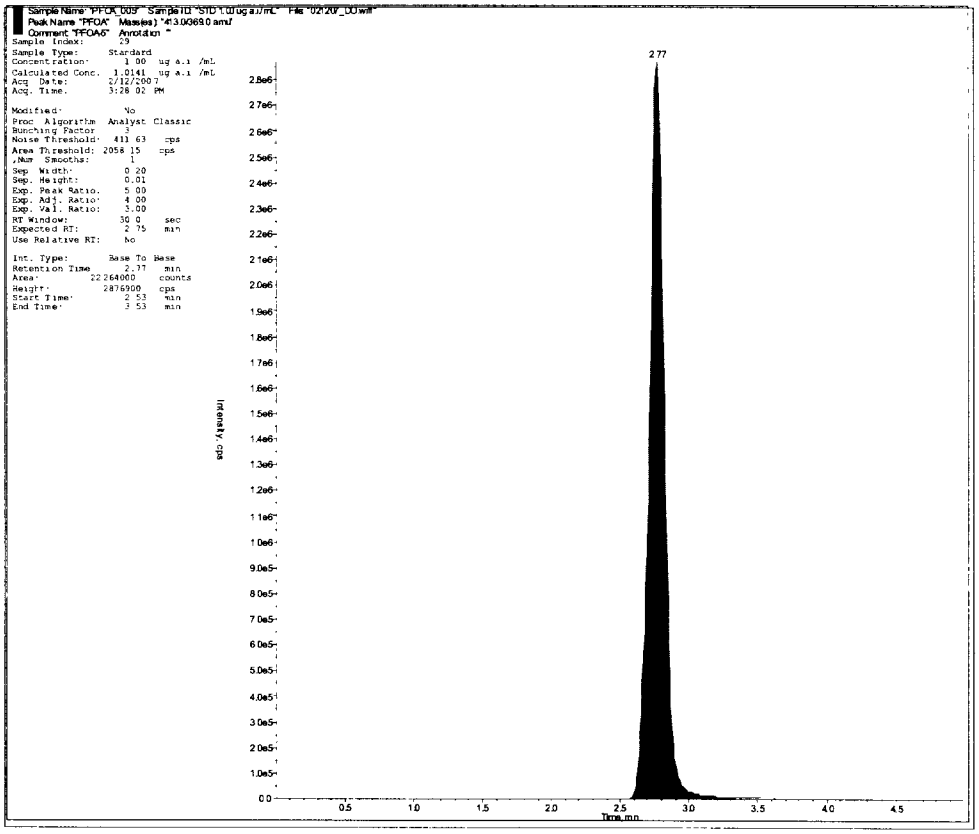
Representative Chromatogram of a Low-level PFOA Calibration Standard



Nominal concentration: 0.100 µg a.i./mL

Appendix 4.8

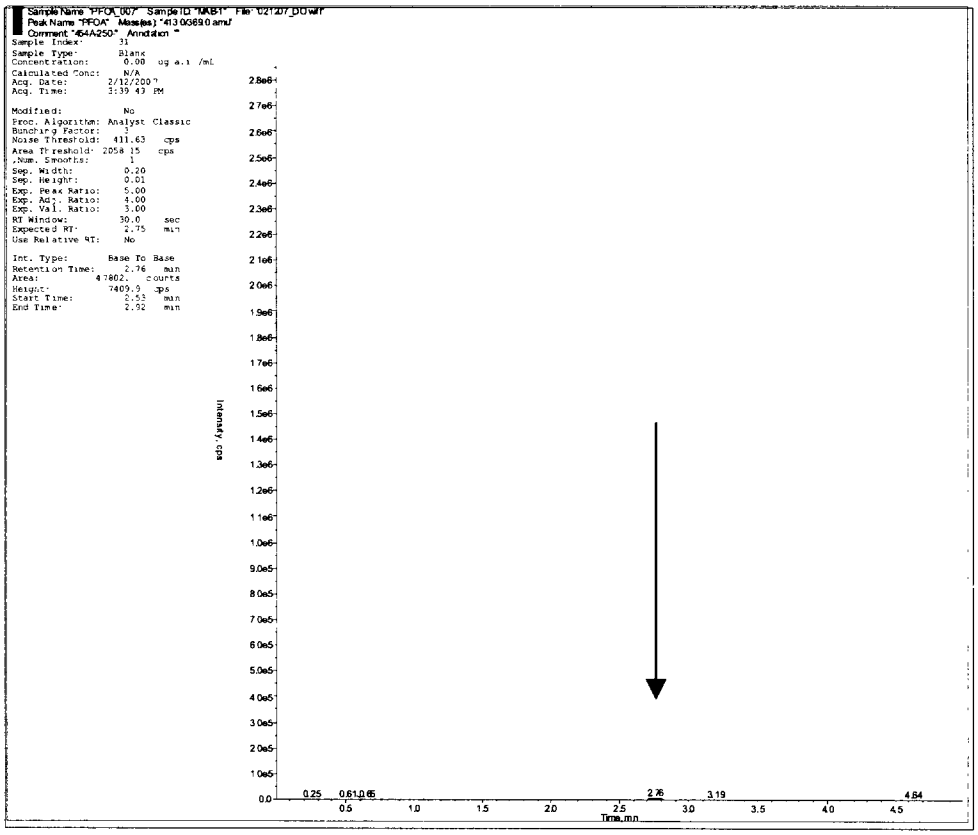
Representative Chromatogram of a High-level PFOA Calibration Standard



Nominal concentration: 1.00 µg a.i./mL

Appendix 4.9

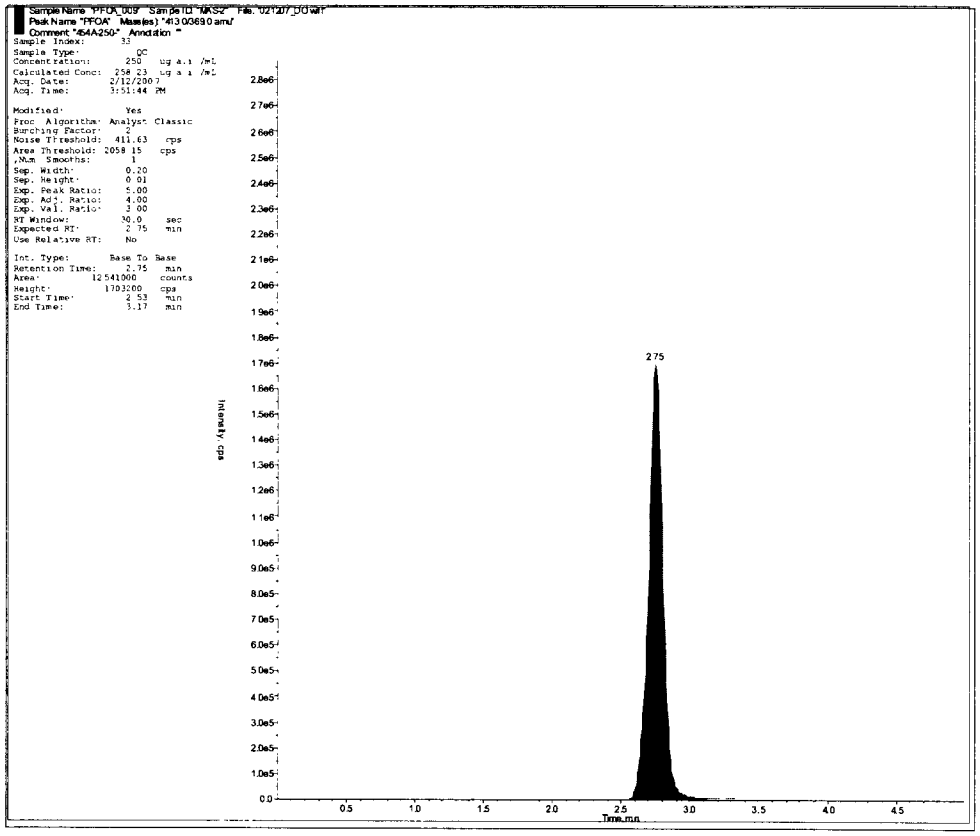
Representative Chromatogram of a Matrix Blank Sample



Sample number 454A-250-MAB-1. Dilution factor = 100X. The arrow indicates the retention time of PFOA.

Appendix 4.10

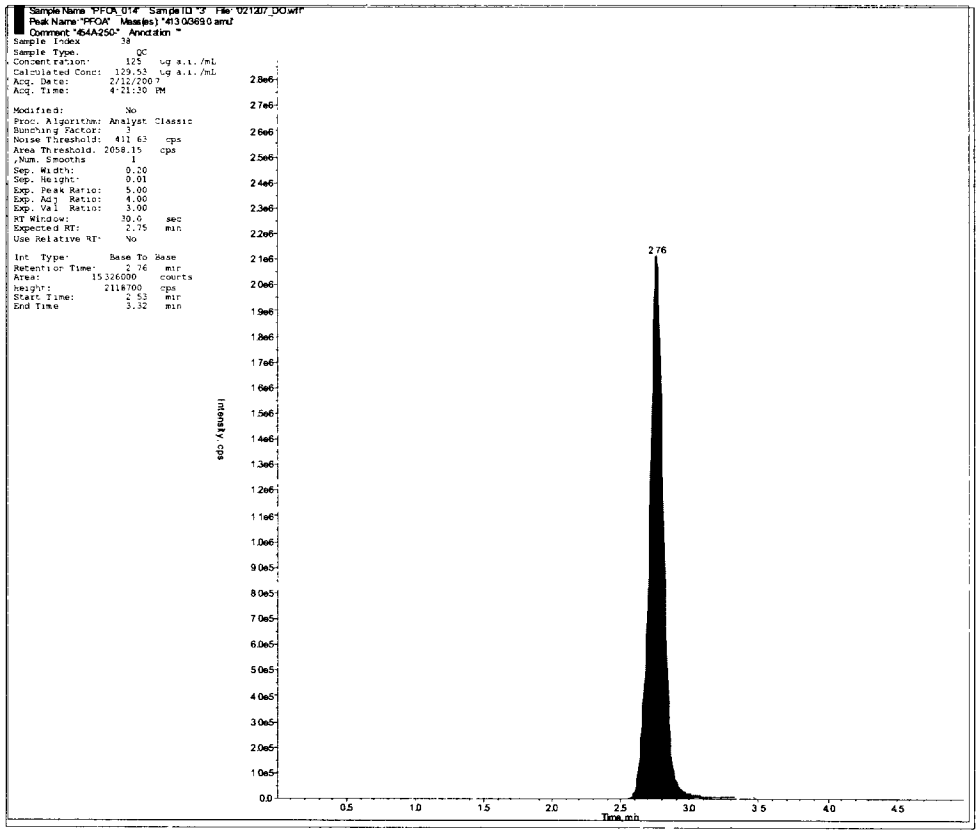
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 454A-250-MAS-2, nominal concentration 250 mg a.i./L. Dilution factor = 100X.

Appendix 4.11

Representative Chromatogram of a Test Sample



Sample number: 454A-250-3, Day 0, nominal concentration 125 mg a.i./L.
Dilution factor = 200X.

Appendix 5

Changes to Protocol

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

1. The protocol was amended to add that the cups will have approximately one-eighth of a teaspoon of sand on the bottom of the cup to provide a monolayer of burrowing substrate for the midges.

2. The protocol was amended to state that the temperature will be 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 protocol was amended to add that the temperature will also be 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. The protocol was amended to state that dissolved oxygen and pH will be measured in samples collected from batches of new test solutions or measured in composite samples of old solutions.

5. The protocol was amended to state that the test concentrations will be 63, 125, 250, 500 and 1000 mg a.i./L rather than 16, 31, 63, 125 and 250 mg a.i./L as requested by the Sponsor to accommodate a no-effect study.

6. The protocol was amended to add the Environmental Laboratory Project Number of E07-0081 as assigned by the Sponsor after the protocol was executed.

7. The protocol was amended to state that test organisms will be obtained from a commercial supplier to complete the protocol as required by GLP.

8. Midge larvae used in the test were obtained from a commercial supplier and held until used in the test rather than hatched from egg masses. This had no adverse impact on the study results.

9. Plastic wrap rather than plastic or glass lids was used to cover the test chambers. This had no adverse impact on the study results.

10. The 24-hour observations were made after 25.25 hours rather than within 24 ± 1 hour of test initiation. This had no adverse impact on the study results.

Appendix 6

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, M.S., Laboratory Supervisor, Aquatics
5. Jon A. MacGregor, B.S., Scientist
6. Susan T. Thomas, B.S., Biologist

Appendix 7

Report Amendment

1.	Original Report:	Pages 1, 2, 3, 4 and 6
	Amendment:	The pages were changed to include the amended report date, the amended report audit date, revised page numbers, and/or new signatures and dates due to the addition of the report amendment as Appendix 7.
	Reason:	To reflect the issuing of an amended report.
2.	Original Report:	Page 9
	Amendment:	The expiration date was changed in the Test Substance section of the report.
	Reason:	A new Certificate of Analysis was supplied after the issuance of the original final report.
3.	Original Report:	Pages 25 and 26
	Amendment:	The Certificate of Analysis was replaced.
	Reason:	A new Certificate of Analysis was supplied after the issuance of the original final report.

AMENDMENT SIGNATURES:

Bill Mindenhout
Study Director

3/8/2007
Date

Laboratory Management

3/8/07
Date

REVIEWED BY:

James H. Coleman
Quality Assurance

3-8-07
Date

CONFIDENTIAL

AMENDED