

AR226-0097

PFOS:
AN EARLY LIFE-STAGE TOXICITY TEST
WITH THE FATHEAD MINNOW (*Pimephales promelas*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-108
3M LAB REQUEST NO. U2723

U.S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1400

OECD Guideline 210

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STUDY INITIATION DATE: July 2, 1999

STUDY COMPLETION DATE: April 26, 2000

Submitted to

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

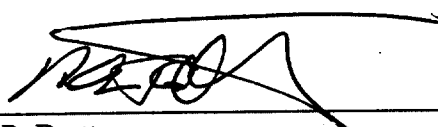
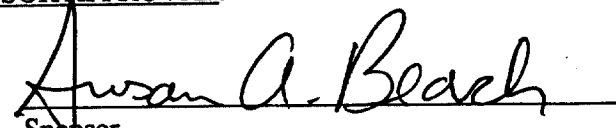
WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-108

STUDY COMPLETION: April 26, 2000

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984, with the following exceptions:

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

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

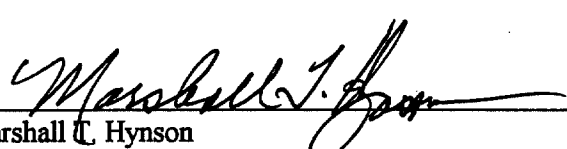
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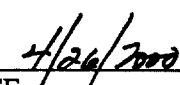
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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	August 2, 1999	August 2, 1999	August 4, 1999
Analytical Sampling, DO Measurements and Egg Separation for Test Initiation	August 5, 1999	August 5, 1999	August 9, 1999
Observations	September 9, 1999	September 9, 1999	September 10, 1999
Analytical Data and Draft Report	December 3, 6 and 7, 1999	December 7, 1999	December 15, 1999
Biological Data and Draft Report	January 3-5, 2000	January 5, 2000	January 7, 2000
Final Report	April 24 and 25, 2000	April 26, 2000	April 26, 2000


Marshall T. Hynson
Quality Assurance Program Supervisor


DATE

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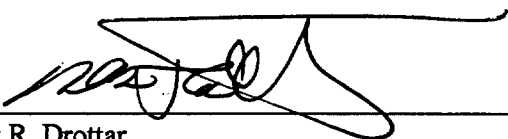
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-108

STUDY DIRECTOR:


Kurt R. Drott
Senior Biologist

4/26/00
DATE

MANAGEMENT:


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Director, Aquatic Toxicology and Non-Target Plants

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SUMMARY

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-108
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: An Early Life-Stage Toxicity Test with the Fathead Minnow (<i>Pimephales promelas</i>)
NOMINAL TEST CONCENTRATIONS:	Negative Control, 0.14, 0.29, 0.57, 1.1, 2.3 and 4.6 mg a.i./L
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 0.15, 0.30, 0.60, 1.2, 2.4 and 4.6 mg a.i./L
TEST DATES:	Experimental Start – August 5, 1999 Biological Termination – September 21, 1999 Experimental Termination – September 22, 1999
LENGTH OF TEST:	47 Days

TEST ORGANISM:	Fathead Minnow (<i>Pimephales promelas</i>)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. Cultures Easton, Maryland 21601
AGE OF TEST ORGANISMS:	Eggs <24 hours old at test initiation

NO-OBSERVED-EFFECT- CONCENTRATION:	0.30 mg a.i./L
LOWEST-OBSERVED-EFFECT- CONCENTRATION:	0.60 mg a.i./L
MAXIMUM ACCEPTABLE TOXICANT CONCENTRATION:	0.42 mg a.i./L

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INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from August 5, 1999 to September 21, 1999. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-108 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the effects of PFOS on early life-stage development of the fathead minnow, *Pimephales promelas*. Time to hatch, hatching success, survival and growth were evaluated during the 47-day study.

EXPERIMENTAL DESIGN

Fathead minnow embryos were exposed to a geometric series of six test concentrations and a negative (dilution water) control under flow-through conditions. Four replicate test chambers were maintained in each treatment and the control group. Each test chamber contained one incubation cup with 20 embryos, resulting in a total of 80 embryos per treatment. The exposure period included a 5-day embryo hatching period, and a 42-day post-hatch juvenile growth period. The Sponsor requested that the study be extended past the normal 28-day post-hatch growth period to determine if survivorship would be affected.

Nominal test concentrations were selected in consultation with the Sponsor, and were based upon acute toxicity data. Nominal test concentrations were 0.14, 0.29, 0.57, 1.1, 2.3 and 4.6 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and the control group at the beginning of the test, on Day 4, at weekly intervals during the test, and at test termination.

To start the test, embryos less than 24 hours old were collected from cultures and groups of 1 and 2 individuals were impartially distributed among incubation cups until each cup contained 20 embryos. One cup was then placed in each treatment and control test chamber. Twice during the next twenty-four hours and daily thereafter, all dead embryos were counted and removed from the cups to avoid contaminating viable embryos. All

eggs that remained were considered viable. Dead embryos continued to be removed daily. After hatching, the larvae were counted and released into the test chambers, where exposure continued until test termination.

Observations of mortality and other clinical signs were made daily during the test. Time to hatch, hatching success, growth, and survival were monitored in each treatment and control group. Data were evaluated to determine the no-observed-effect-concentration (NOEC), the lowest-observed-effect-concentration (LOEC) and the maximum acceptable toxicant concentration (MATC).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "PFOS: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1400 (1); *OECD Guideline for Testing of Chemicals, 210: Fish Early-Life Stage Toxicity Test* (2); ASTM Standard E1241-88, *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fish* (3) and *Standard Evaluation Procedure, Fish Early Life-Stage Test* (4).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 90.49%, and an expiration date of 2008. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

One stock solution was prepared for each of the six concentrations tested. A primary stock solution was prepared by dissolving PFOS in dilution water at a concentration of 88.4 mg a.i./L. The primary stock solution was stirred with an electric stainless steel top-down mixer until all the test substance was dissolved. The primary stock solution was proportionally diluted with dilution water to prepare five secondary stock solutions at concentrations of 44.2, 22.1, 11.0, 5.52 and 2.76 mg a.i./L. Stock solutions were prepared every three to four days during the test. The five stocks were injected into the diluter mixing chambers (at a rate of 6.0 mL/minute)

where they were mixed with dilution water (at a rate of 116 mL/minute) to achieve the desired test concentrations. The resultant test concentrations were adjusted for purity of the active ingredient in the test substance (90.49%).

Test Organism

The fathead minnow, *Pimephales promelas*, was selected as the test species for this study. Fathead minnows are representative of an important group of aquatic vertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. Fathead minnow embryos used in this test originated from cultures maintained by Wildlife International Ltd., Easton, Maryland. The embryos were removed from spawning substrates and examined under the dissecting microscope to select healthy specimens at approximately the same stage of development. Embryos collected for use in the test were from six individual spawns and were less than 24 hours old when the test was initiated.

Newly-hatched larvae were fed live brine shrimp nauplii (*Artemia sp.*) 3 times per day during the first 7 days post-hatch. On Days 8 through 40 post-hatch, all fish were fed live brine shrimp nauplii 3 times daily on weekdays and 2 times daily on weekends. Brine shrimp nauplii were obtained by hatching cysts purchased from Bonneville Artemia International Inc., Salt Lake City, Utah. Fish were not fed for at least 48 hours prior to the termination of the test to allow for clearance of the digestive tracts before weight measurements were made. To ensure that the feeding rate per fish remained constant, rations were adjusted each week to account for losses due to mortality.

The average wet weight of 16 fish in the negative control (Replicate A) measured at the end of the test was 0.16 g. Biomass loading (the total wet weight of the fish per liter of test water) at the end of the test was 0.062 g of fish per liter of test water that passed through the test chamber during a 24-hour period. Instantaneous loading (the total wet weight of fish per liter of water in the tank) at the end of the test was 0.37 g of fish per liter of test water.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance and a negative (dilution water) control. A peristaltic pump (Cole-Parmer Instrument Company) was used to deliver the six test substance stock solutions into mixing chambers assigned to each treatment group. The stock solutions were diluted with well water in the mixing chambers in order to obtain the desired test concentrations. The flow of

dilution water to the mixing chambers was controlled by rotameters. Rotameters were calibrated prior to test initiation and at weekly intervals thereafter. The flow of test water from each mixing chamber was split and allowed to flow into four replicate test chambers. The proportion of the test water that was split into each replicate was checked prior to the test, and at weekly intervals thereafter to ensure that flow rates varied by no more than $\pm 10\%$ of the mean of the four replicates.

The diluter was adjusted so that each test chamber received approximately 6.0 volume additions of test water every 24 hours. The test substance delivery pump was calibrated before the test and at weekly intervals thereafter. The general operation of the diluter was checked visually at least two times per day during the test and at least once at the beginning and end of the test.

The test chambers were 9-L glass aquaria filled with approximately 7 L of test solution. The depth of the test water in a representative test chamber was approximately 17 cm. The test chambers were impartially positioned in a temperature-controlled environmental chamber designed to maintain a temperature of $25 \pm 2^\circ\text{C}$. Test chambers were labeled with the project number, test concentration and replicate.

The embryo incubation cups were suspended in the water column of each test chamber and attached to a rocker arm. The reciprocating motion of the rocker arm (approximately 2 rpm) facilitated circulation of test water around the embryos during incubation. The incubation cups were constructed from glass cylinders approximately 50 mm in diameter with 425 μm nylon screen mesh attached to the bottom with silicone sealant.

Dilution Water

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

The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to delivery to the diluter system, the water again was filtered (0.45 μm), then passed through a UV sterilizer to remove

microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix II.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark 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 was measured at the surface of the negative control (replicate A) at test initiation, at test termination and at weekly intervals during the test. Light intensity ranged from 581 to 619 lux.

Temperature was measured in each test chamber at the beginning and end of the test and at weekly intervals during the test using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was $25 \pm 2^\circ\text{C}$. The pH was measured at the beginning and end of the test and at weekly intervals during the test in alternating replicates of each treatment and the control group. Dissolved oxygen concentrations were measured daily in alternating replicates of each treatment and the control group during the first 7 days of the test, at weekly intervals during the test, and at test termination. Hardness, alkalinity and specific conductance were measured in the negative control and the highest treatment group with surviving organisms at the beginning of the test, once a week during the test, and at test termination.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity were measured based on procedures in *Standard Methods for the Examination of Water and Wastewater* (5).

Biological Observations and Measurements

Observations of mortality were made twice during the first 24 hours of the embryo exposure period and daily until hatch. After hatching, larvae were observed daily to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior during the 42-day post-hatch exposure

period. From these observations, hatching success, time to hatch, and post-hatch growth and survival were evaluated. Hatching success was estimated as the percentage of viable embryos that hatched successfully. Post-hatch survival was estimated as the number of larvae surviving to test termination divided by the number of embryos which hatched successfully. Post-hatch growth of the fathead minnows was evaluated at the conclusion of the 42-day post-hatch exposure period. Total lengths for each surviving fish were determined using a digitizing tablet with the SIGMA SCAN™ scientific measurement system and wet and dry weights were measured using an analytical balance.

Data Analyses

Hatching success, post-hatch survival, wet weight, dry weight and total length of the juvenile fish were test endpoints that were analyzed statistically. Data collected during this test were either continuous-variable data (e.g., weight and length) or discrete-variable data (e.g., mortality proportions).

Discrete-variable data were analyzed using 2 X 2 contingency tables to identify treatment groups that showed a statistically significant difference ($p \leq 0.05$) from the negative control group. All continuous-variable data were evaluated for normality using Shapiro-Wilk's test and for homogeneity of variance using Bartlett's test. Analysis of variance and Dunnett's test were used to evaluate differences between treatment and control means.

The results of the statistical analyses were used to aid in the determination of the NOEC, LOEC and MATC. The MATC was estimated as the geometric mean of the NOEC and LOEC. All statistical tests were performed on a personal computer using TOXSTAT 3.5 (6) and SPSS/PC Version 2.0 (7) statistical software.

Analytical Chemistry

Prior to test initiation, one set of pretest water samples was collected from two replicate test chambers of each treatment group and the negative control to evaluate diluter performance. On Days 0, 4, 7, 14, 21, 28, 35, 42 and at test termination, samples were collected from two alternating replicate test chambers of each treatment and the control group to measure concentrations of the test substance. Samples were collected at mid-depth from each test chamber. Samples were collected in 30-mL Nalgene® bottles and analyzed immediately. Analytical procedures used in the analysis of the samples are provided in Appendix III.

RESULTS AND DISCUSSION

Verification of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 0.14, 0.29, 0.57, 1.1, 2.3 and 4.6 mg a.i./L. When measured concentrations of samples analyzed during the test were averaged, the mean measured concentrations were 0.15, 0.30, 0.60, 1.2, 2.4 and 4.6 mg a.i./L, which represented 107, 103, 105, 109, 104 and 100% of the nominal concentrations, respectively. Mean measured concentrations were used to express the NOEC, LOEC and MATC. Pre-test samples collected to verify diluter operation (Appendix III) were not used to calculate mean measured values.

Physical and Chemical Measurements of Water

Measurements of the specific conductance, hardness and alkalinity made on the negative control and treatment water are presented in Table 2. No apparent differences in these parameters existed between the treatments and the control group. Measurements of pH, dissolved oxygen, and temperature are presented in Tables 3, 4, and 5, respectively. Water pH measurements were consistent over time and showed no obvious differences between control water and the treatment groups. Measurements of pH ranged from 8.0 to 8.4. Dissolved oxygen concentrations remained ≥ 7.6 mg/L (92% of saturation). All temperature measurements were within the desired range of $25 \pm 2^\circ\text{C}$.

Hatching Success and Time to Hatch

All viable fathead minnow embryos hatched on Day 4 or 5 of the test (Table 6). There were no apparent differences between the time to hatch in the negative control and the PFOS treatment groups. Hatching success in the negative control was 93%. Hatching success in the PFOS treatment groups was $\geq 90\%$. Hatching success was not significantly different in any PFOS treatment group in comparison to the negative control ($p > 0.05$).

Post-Hatch Survival and Clinical Observations

After 42 days of post-hatch exposure, survival in the negative control was 88% (Table 7). Post-hatch survival in the 0.15, 0.30, 0.60, 1.2, 2.4 and 4.6 mg a.i./L treatment groups was 79, 81, 66, 5.4, 0 and 0%, respectively. Analysis of post-hatch survival data by 2 X 2 contingency tables showed that survival was significantly different in the 0.60, 1.2, 2.4 and 4.6 mg a.i./L treatment groups in comparison to the negative

control ($p \leq 0.05$). All fathead minnows surviving to test termination appeared normal with no overt signs of sublethal toxicity. Fathead minnows which did not survive generally appeared to be swimming erratically prior to death (Appendix IV).

Growth

Growth data for each treatment and the control group are summarized in Table 8. The individual measurements are provided in Appendices V, VI and VII. Growth was evaluated at test termination by measuring the total length, wet weight and dry weight of each surviving fish. The statistical analyses of growth data did not include PFOS treatment groups which had a statistically significant effect on survival. Fathead minnows exposed to PFOS at concentrations of 0.15 or 0.30 mg a.i./L for 42 days post-hatch showed no statistically significant reductions in total length, wet weight or dry weight in comparison to the negative control ($p > 0.05$).

CONCLUSIONS

Fathead minnows (*Pimephales promelas*) exposed to PFOS at concentrations ≤ 0.30 mg a.i./L for 42 days post-hatch showed no statistically significant reductions in time to hatch, hatching success, survival or growth. The most sensitive endpoint in this study was post-hatch survival. Fathead minnows exposed to PFOS at concentrations ≥ 0.60 mg a.i./L had statistically significant differences in post-hatch survival in comparison to the negative control. Consequently, the LOEC for this study, based on post-hatch survival, was 0.60 mg a.i./L. The NOEC was 0.30 mg a.i./L and the MATC was calculated to be 0.42 mg a.i./L.

REFERENCES

- 1 U.S. Environmental Protection Agency. 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1400: *Fish Early-Life Stage Toxicity Test*.
- 2 OECD Guideline for Testing of Chemicals. 1992. *Guideline 210: Fish Early-Life Stage Toxicity Test*.
- 3 ASTM Standard E1241-88. 1988. *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fish*. American Society for Testing and Materials.
- 4 U.S. Environmental Protection Agency. 1986. *Standard Evaluation Procedure, Fish Early Life-Stage Test*. Office of Pesticide Programs. Hazard Evaluation Division. EPA 540/9-86-138.
- 5 APHA, AWWA, WPCF. 1989. *Standard Methods for the Examination of Water and Wastewater*. 17th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 West, Inc. and D.D. Gulley. 1996. TOXSTAT® 3.5. Western EcoSystems Technology, Inc. Cheyenne, Wyoming.
- 7 SPSS Inc. 1988. SPSS/PC+ Version 2.0. Chicago, Illinois.

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Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation										
Test Substance:	PFOS										
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>										
Dilution Water:	Well Water										
Nominal Test Concentration (mg a.i./L)	Day 0	Day 4	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 47	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	<LOQ ¹ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ <LOQ	<LOQ	-
0.14	0.147 0.160	0.141 0.140	0.144 0.148	0.134 0.135	0.153 0.143	0.160 0.158	0.179 0.173	0.157 0.160	0.147 0.155	0.15	107
0.29	0.287 0.277	0.270 0.289	0.292 0.296	0.269 0.266	0.307 0.315	0.343 0.341	0.311 0.325	0.319 0.313	0.296 0.276	0.30	103
0.57	0.571 0.576	0.619 0.659	0.597 0.642	0.539 0.535	0.608 0.580	0.639 0.617	0.646 0.644	0.575 0.576	0.545 0.543	0.60	105
1.1	1.14 1.13	1.21 1.25	1.13 1.23	1.03 1.10	1.19 1.24	1.30 1.31	1.30 1.31	1.14 1.19	1.13 1.09	1.2	109
2.3	2.21 2.27	2.52 2.46	2.43 2.38	- ² - ²	- -	- -	- -	- -	- -	2.4	104
4.6	4.56 4.40	4.79 4.79	4.46 4.76	- ² - ²	- -	- -	- -	- -	- -	4.6	100

¹The limit of quantitation (LOQ) was 0.0458 mg a.i./L.

²Measurements discontinued due to 100% mortality.

¹The limit of quantitation (LOQ) was 0.0458 mg a.i./L.²Measurements discontinued due to 100% mortality.

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Table 2

Specific Conductance, Hardness and Alkalinity of the Test Solutions

Sponsor: 3M Corporation									
Test Substance: PFOS									
Test Organism: Fathead Minnow, <i>Pimephales promelas</i>									
Dilution Water: Well Water									
Parameter	Test Group	Test Day							
		0	7	14	21	28	35	42	47
Specific Conductance (μ mhos/cm)	Negative Control	315	330	300	300	290	300	290	290
	PFOS Treatment	330 ¹	310 ²	315 ³	300 ³	300 ³	300 ³	300 ³	315 ³
Hardness (mg/L as CaCO ₃)	Negative Control	136	140	140	136	144	136	140	136
	PFOS Treatment	140 ¹	140 ²	144 ³	140 ³	136 ³	140 ³	140 ³	136 ³
Alkalinity (mg/L as CaCO ₃)	Negative Control	176	178	178	178	178	176	178	174
	PFOS Treatment	178 ¹	178 ²	176 ³	178 ³	176 ³	176 ³	180 ³	176 ³
Test Chamber Replicate		A	D	A	B	C	A	B	C
¹ 4.6 mg a.i./L									
² 2.4 mg a.i./L									
³ 1.2 mg a.i./L									

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Table 3

pH of Water in the Test Chambers

Sponsor:	3M Corporation							
Test Substance:	PFOS							
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>							
Dilution Water:	Well Water							
Mean Measured Test Concentration (mg a.i./L)	Test Day							
	0	7	14	21	28	35	42	47
Negative Control	8.1	8.2	8.2	8.3	8.4	8.0	8.1	8.1
0.15	8.2	8.2	8.2	8.3	8.4	8.0	8.1	8.1
0.30	8.2	8.2	8.2	8.3	8.4	8.1	8.1	8.2
0.60	8.2	8.2	8.2	8.3	8.4	8.1	8.1	8.2
1.2	8.2	8.2	8.2	8.3	8.4	8.0	8.2	8.2
2.4	8.2	8.2	- ¹	-	-	-	-	-
4.6	8.2	8.2	- ¹	-	-	-	-	-
Replicate	A	D	A	B	C	A	B	C
¹ Measurements discontinued due to 100% mortality.								

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Table 4

Dissolved Oxygen Content (mg/L) of Water in the Test Chambers¹

Sponsor:	3M Corporation													
Test Substance:	PFOS													
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>													
Dilution Water:	Well Water													
Mean Measured Test Concentration (mg a.i./L)	Test Day													
	0	1	2	3	4	5	6	7	14	21	28	35	42	47
Negative Control	8.1	8.0	8.1	8.1	8.0	8.0	8.0	8.2	8.1	8.1	8.2	7.8	7.6	8.0
0.15	8.1	8.0	8.1	8.1	8.0	8.0	8.0	8.2	8.2	8.1	8.2	7.6	7.8	8.0
0.30	8.1	8.1	8.2	8.1	8.0	8.0	7.9	8.1	8.2	8.1	8.2	7.6	7.8	8.0
0.60	8.1	8.0	8.2	8.1	8.1	8.0	8.0	8.1	8.1	8.1	8.2	7.8	7.6	8.0
1.2	8.2	8.0	8.1	8.0	8.0	7.9	8.0	8.2	8.2	8.0	8.2	7.6	7.9	8.2
2.4	8.0	8.0	8.1	8.0	8.0	8.1	8.0	8.2	-- ²	--	--	--	--	--
4.6	8.1	8.0	8.1	8.1	8.1	8.0	8.0	8.1	-- ²	--	--	--	--	--
Replicate	A	B	C	D	A	B	C	D	A	B	C	A	B	C

¹A dissolved oxygen concentration of 4.9 mg/L represents 60% saturation at 25°C in freshwater.²Measurements discontinued due to 100% mortality.

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Table 5

Temperature (°C) of Water in the Test Chambers

Sponsor:		3M Corporation							
Test Substance:		PFOS							
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>							
Dilution Water:		Well Water							
Mean Measured Test Concentration (mg a.i./L)	Replicate	Test Day							
		0	7	14	21	28	35	42	47
Negative Control	A ¹	24.4	24.7	24.7	24.5	24.5	24.7	24.5	24.6
	B	24.4	24.7	24.7	24.5	24.5	24.7	24.4	24.5
	C	24.4	24.7	24.6	24.6	24.5	24.7	24.5	24.5
	D	24.5	24.7	24.7	24.5	24.5	24.7	24.5	24.5
0.15	A	24.4	24.7	24.7	24.6	24.4	24.7	24.5	24.4
	B	24.5	24.6	24.7	24.6	24.5	24.8	24.6	24.4
	C	24.4	24.7	24.7	24.6	24.5	24.7	24.5	24.5
	D	24.5	24.7	24.6	24.5	24.5	24.7	24.4	24.4
0.30	A	24.4	24.7	24.7	24.6	24.5	24.8	24.4	24.4
	B	24.4	24.7	24.7	24.5	24.5	24.7	24.4	24.4
	C	24.4	24.7	24.6	24.5	24.4	24.7	24.5	24.4
	D	24.5	24.7	24.6	24.5	24.5	24.7	24.4	24.4
0.60	A	24.4	24.7	24.6	24.5	24.4	24.6	24.4	24.4
	B	24.4	24.6	24.5	24.4	24.4	24.7	24.3	24.3
	C	24.3	24.6	24.6	24.5	24.4	24.6	24.3	24.4
	D	24.3	24.6	24.5	24.4	24.4	24.6	24.4	24.4
1.2	A	24.5	24.5	24.5	24.4	24.4	24.5	24.3	24.3
	B	24.4	24.6	24.5	24.5	24.5	24.6	24.4	24.4
	C	24.4	24.6	24.6	24.5	24.5	24.6	24.4	24.4
	D	24.5	24.7	24.6	24.5	24.5	24.6	24.5	24.4
2.4	A	24.5	24.7	-- ²	--	--	--	--	--
	B	24.6	24.7	-- ²	--	--	--	--	--
	C	24.6	24.7	-- ²	--	--	--	--	--
	D	24.5	24.7	-- ²	--	--	--	--	--
4.6	A	24.5	24.6	-- ²	--	--	--	--	--
	B	24.4	24.6	-- ²	--	--	--	--	--
	C	24.5	24.5	-- ²	--	--	--	--	--
	D	24.5	24.5	-- ²	--	--	--	--	--

¹A continuous recording of temperature in the negative control replicate A showed that the temperature ranged from 24.0 to 25.0°C throughout the test.

²Measurements discontinued due to 100% mortality.

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Table 6
Hatching Success of Fathead Minnows Exposed to the Test Substance

Sponsor:	3M Corporation								
Test Substance:	PFOS								
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>								
Dilution Water:	Well Water								
Mean Measured Test Concentration (mg a.i./L)	Replicate	Number of Eggs Exposed	Number Hatched					Total Number Hatched	Treatment Percent Hatching Success ¹
			Day 1	Day 2	Day 3	Day 4	Day 5		
Negative Control	A	20	0	0	0	7	18	74	93
	B	20	0	0	0	5	18		
	C	20	0	0	0	6	20		
	D	20	0	0	0	2	18		
0.15	A	20	0	0	0	8	18	76	95
	B	20	0	0	0	5	19		
	C	20	0	0	0	2	19		
	D	20	0	0	0	3	20		
0.30	A	20	0	0	0	4	17	72	90
	B	20	0	0	0	1	18		
	C	20	0	0	0	2	18		
	D	20	0	0	0	7	19		
0.60	A	20	0	0	0	5	18	76	95
	B	20	0	0	0	6	19		
	C	20	0	0	0	10	20		
	D	20	0	0	0	7	19		
1.2	A	20	0	0	0	9	18	74	93
	B	20	0	0	0	7	19		
	C	20	0	0	0	4	18		
	D	20	0	0	0	5	19		
2.4	A	20	0	0	0	7	18	75	94
	B	20	0	0	0	3	19		
	C	20	0	0	0	4	20		
	D	20	0	0	0	2	18		
4.6	A	20	0	0	0	3	19	74	93
	B	20	0	0	0	4	17		
	C	20	0	0	0	1	19		
	D	20	0	0	0	6	19		

¹ Percent Hatching Success = $\frac{\text{Number of Eggs Hatched}}{\text{Number of Eggs Exposed}} \times 100$

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Table 7

Survival of Fathead Minnow Larvae Exposed to the Test Substance for 42 Days Post-Hatch

Sponsor:		3M Corporation								
Test Substance:		PFOS								
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>								
Dilution Water:		Well Water								
Mean Measured Test Concentration (mg a.i./L)	Replicate	Initial Number of Larvae	Approximate Live Counts ¹ (Days Post-Hatch)						Replicate Percent Survival	Treatment Percent Survival
			7	14	21	28	35	42		
Negative Control	A	18	17	17	17	17	16	16	89	88
	B	18	15	15	15	15	15	15	83	
	C	20	19	18	18	17	17	17	85	
	D	18	18	18	18	18	17	17	94	
0.15	A	18	17	17	16	15	15	15	83	79
	B	19	18	18	17	15	15	15	79	
	C	19	17	16	16	15	15	15	79	
	D	20	17	17	16	15	15	15	75	
0.30	A	17	15	15	15	15	15	15	88	81
	B	18	18	17	16	16	16	16	89	
	C	18	15	15	15	13	11	11	61	
	D	19	19	18	18	16	16	16	84	
0.60	A	18	17	16	16	15	13	12	67	66*
	B	19	17	17	16	14	14	13	68	
	C	20	19	19	18	13	13	13	65	
	D	19	18	18	16	13	13	12	63	
1.2	A	18	12	12	7	3	3	2	11	5.4*
	B	19	11	10	4	2	2	1	5.3	
	C	18	10	7	2	1	1	1	5.6	
	D	19	7	6	0	0	0	0	0.0	
2.4	A	18	0	0	0	0	0	0	0.0	0.0*
	B	19	0	0	0	0	0	0	0.0	
	C	20	0	0	0	0	0	0	0.0	
	D	18	0	0	0	0	0	0	0.0	
4.6	A	19	0	0	0	0	0	0	0.0	0.0*
	B	17	0	0	0	0	0	0	0.0	
	C	19	0	0	0	0	0	0	0.0	
	D	19	0	0	0	0	0	0	0.0	

¹Live counts made on Day 2 through 21 post-hatch were variable due to the difficulty in counting large numbers of living fish. The counts made on Day 42 were exact counts of all fish surviving at the end of the test.

*Indicates a significant difference from the negative control using a 2 X 2 contingency table ($p \leq 0.05$).

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Table 8

Total Length, Wet Weight and Dry Weight of Fathead Minnow Larvae
at the End of the 42-Day Post-Hatch Observation Period

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
Mean Measured Test Concentration (mg a.i./L)	Number of Surviving Larvae	Total Length (mm) Mean ($\pm$ SD)	Wet Weight (mg) Mean ($\pm$ SD)	Dry Weight (mg) Mean ($\pm$ SD)
Negative Control	65	26.5 ($\pm$ 0.721)	158 ($\pm$ 9.10)	32.5 ($\pm$ 1.20)
0.15	60	26.6 ($\pm$ 0.208)	160 ($\pm$ 3.10)	33.3 ($\pm$ 0.900)
0.30	58	26.6 ($\pm$ 0.813)	167 ($\pm$ 11.9)	34.2 ($\pm$ 2.70)
0.60 ¹	50	26.5 ($\pm$ 0.399)	166 ($\pm$ 11.3)	33.5 ($\pm$ 2.70)
1.2 ¹	4	26.7 ($\pm$ 2.02)	185 ($\pm$ 33.8)	35.4 ($\pm$ 6.66)
2.4 ¹	0	--	--	--
4.6 ¹	0	--	--	--
¹ Not analyzed statistically due to a significant ($p \leq 0.05$) effect on survival.				

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APPENDIX I

Dilution Water Chemistry Parameters Measured During the
4-Week Period Immediately Preceding the Test

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>
Dilution Water:	Well Water

	Mean	Range
Specific Conductance (μ mhos/cm)	321 (N = 4)	315 - 330
Hardness (mg/L as CaCO ₃)	126 (N = 4)	124 - 128
Alkalinity (mg/L as CaCO ₃)	172 (N = 4)	170 - 172
pH	8.2 (N = 4)	8.2 - 8.3

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APPENDIX II
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Well Water¹

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

¹ Analyses performed by OST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.

² Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.

³ Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

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APPENDIX III

THE ANALYSIS OF PFOS IN FRESHWATER
IN SUPPORT OF
WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-108

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REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: An Early Life-Stage Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-108

PRINCIPAL INVESTIGATOR:


Don A. MacGregor, B.S.
Scientist

4/26/00
DATE

MANAGEMENT:


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

4/26/00
DATE

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Introduction

Freshwater samples were collected from an early life-stage toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the fathead minnow (*Pimephales promelas*). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-108. The analyses of these water samples were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis between August 4, 1999 and September 21, 1999 and were typically analyzed on each sample receipt day.

Test Substance and Internal Standard

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

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

Analytical Method

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

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

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-

Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C₁₈ analytical column (50 mm x 2 mm I.D., 3 µm particle size). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.000915 to 0.00915 mg a.i./L, were analyzed with the samples. The same and most prominent peak response for PFOS was utilized to monitor PFOS in all calibration, quality control, and study samples. No attempt was made to quantify PFOS on the basis of individual isomeric components. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentration ratios (PFOS : internal standard) of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.0458 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

Matrix Blank and Fortification Samples

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

Freshwater was fortified at 0.0915, 0.915 and 7.32 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected for the mean procedural recovery of 102%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-108-3, nominal concentration of 0.14 mg a.i./L in freshwater.

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

Concentration Ratio = Concentration of Analyte/Concentration of Internal Standard

Internal Standard Concentration: 0.100 mg/L

Initial Volume: 0.500 mL

Final Volume: 25.0 mL

Dilution Factor: 50.0

PFOS Peak Area: 244330

Internal Standard Peak Area: 2316753

Peak Area Ratio: 0.10546

Calibration curve equation.

Slope: 3.44236

Intercept: 0.00427

Curve is weighted (1/x)

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

$$= \frac{0.10546 - 0.00427}{3.44236} \times 0.100$$

$$= 0.00294$$

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

$$= 0.00294 \times 50.0$$

$$= 0.147$$

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$$\begin{aligned}\text{Percent of Nominal Concentration} &= \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100 \\ &= \frac{0.147}{0.14} \times 100 \\ &= 105\%\end{aligned}$$

RESULTS

Sample Analysis

Freshwater samples were collected from the early life-stage toxicity study with the fathead minnow (*Pimephales promelas*) at pretest, August 4, 1999, at test initiation, August 5, 1999 (Day 0), on August 9, 1999 (Day 4), on August 12, 1999 (Day 7) and weekly throughout the test until test termination on September 21, 1999 (Day 47). The measured concentrations of PFOS in the samples collected at pre-test ranged from 91.4 to 105% of the nominal concentrations (Table 4). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 95.5 to 114% of the nominal concentrations (Table 5). Samples collected at Day 4, Day 7, Day 14, Day 21, Day 28, Day 35 and Day 42 had measured concentration ranges of 93.1 to 116%, 97.0 to 113%, 91.7 to 100%, 102 to 113%, 108 to 119%, 107 to 128% and 101 to 114% of nominal values, respectively. Samples collected at test termination (Day 47) had a measured concentration range of 95.2 to 111% of nominal values. A representative ion chromatogram of a test sample is shown in Figure 7.

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Table 1

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (50 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	25.0 µL
PFOS RETENTION TIME:	Approximately 4.3 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 3.0 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

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Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of
Number (454A-108-)	Type	PFOS ¹ (mg a.i./L)
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ
MAB-4	Matrix Blank	< LOQ
MAB-5	Matrix Blank	< LOQ
MAB-6	Matrix Blank	< LOQ
MAB-7	Matrix Blank	< LOQ
MAB-8	Matrix Blank	< LOQ
MAB-9	Matrix Blank	< LOQ

¹ The limit of quantitation (LOQ) was 0.0458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

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Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-108-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered
	Fortified	Measured	
MAS-1	0.0915	0.0908	99.2
MAS-4	0.0915	0.0924	101
MAS-7	0.0915	0.0893	97.6
MAS-10	0.0915	0.0942	103
MAS-13	0.0915	0.0884	96.6
MAS-16	0.0915	0.0886	96.8
MAS-19	0.0915	0.103	113
MAS-22	0.0915	0.0933	102
MAS-25	0.0915	0.0970	106
MAS-2	0.915	0.894	97.7
MAS-5	0.915	0.908	99.2
MAS-8	0.915	0.899	98.3
MAS-11	0.915	0.961	105
MAS-14	0.915	0.988	108
MAS-17	0.915	0.912	99.7
MAS-20	0.915	0.988	108
MAS-23	0.915	0.909	99.3
MAS-26	0.915	0.933	102
MAS-3	7.32	7.59	104
MAS-6	7.32	7.53	103
MAS-9	7.32	7.75	106
MAS-12	7.32	7.33	100
MAS-15	7.32	7.66	105
MAS-18	7.32	7.36	101
MAS-21	7.32	7.51	103
MAS-24	7.32	7.47	102
MAS-27	7.32	7.13	97.4
Mean = 102			
Standard Deviation = 3.99			
CV = 3.91%			
N = 27			

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Table 4

Measured Concentrations of PFOS in Pre-test Diluter Verification Samples from an Early Life-Stage Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-108-)	Sampling Time (Hours)	PFOS Measured Concentration (mg a.i./L) ^{1,2}	Percent of Nominal
0.0 (Negative Control)	PT-1	-24	< LOQ	--
	PT-2	-24	< LOQ	--
0.14	PT-3	-24	0.141	101
	PT-4	-24	0.147	105
0.29	PT-5	-24	0.276	95.2
	PT-6	-24	0.265	91.4
0.57	PT-7	-24	0.565	99.1
	PT-8	-24	0.561	98.4
1.1	PT-9	-24	1.10	100
	PT-10	-24	1.09	99.1
2.3	PT-11	-24	2.13	92.6
	PT-12	-24	2.17	94.3
4.6	PT-13	-24	4.48	97.4
	PT-14	-24	4.47	97.2

¹ The limit of quantitation (LOQ) was 0.0458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

² Measured values of matrix blanks and matrix fortification samples analyzed concurrently with pre-test samples were <LOQ, 101, 97.7 and 112 percent of nominal concentrations.

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Table 5

Measured Concentrations of PFOS in Freshwater Samples from a
Fathead Minnow Early Life-Stage Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-108-)	Sampling Time (Day)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
0.0 (Negative Control)	1	0	< LOQ	--
	2	0	< LOQ	--
	15	4	< LOQ	--
	16	4	< LOQ	--
	29	7	< LOQ	--
	30	7	< LOQ	--
	43	14	< LOQ	--
	44	14	< LOQ	--
	53	21	< LOQ	--
	54	21	< LOQ	--
	63	28	< LOQ	--
	64	28	< LOQ	--
	73	35	< LOQ	--
	74	35	< LOQ	--
	83	42	< LOQ	--
	84	42	< LOQ	--
	93	47	< LOQ	--
	94	47	< LOQ	--
0.14	3	0	0.147	105
	4	0	0.160	114
	17	4	0.141	101
	18	4	0.140	100
	31	7	0.144	103
	32	7	0.148	106
	45	14	0.134	95.7
	46	14	0.135	96.4
	55	21	0.153	109
	56	21	0.143	102
	65	28	0.160	114
	66	28	0.158	113
	75	35	0.179	128
	76	35	0.173	124
	85	42	0.157	112
	86	42	0.160	114
	95	47	0.147	105
	96	47	0.155	111

¹ The limit of quantitation (LOQ) was 0.0458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

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Table 5 (continued)

Measured Concentrations of PFOS in Freshwater Samples from a
Fathead Minnow Early Life-Stage Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-108-)	Sampling Time (Day)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
0.29	5	0	0.287	99.0
	6	0	0.277	95.5
	19	4	0.270	93.1
	20	4	0.289	99.7
	33	7	0.292	101
	34	7	0.296	102
	47	14	0.269	92.8
	48	14	0.266	91.7
	57	21	0.307	106
	58	21	0.315	109
	67	28	0.343	118
	68	28	0.341	118
	77	35	0.311	107
	78	35	0.325	112
	87	42	0.319	110
	88	42	0.313	108
	97	47	0.296	102
	98	47	0.276	95.2
0.57	7	0	0.571	100
	8	0	0.576	101
	21	4	0.619	109
	22	4	0.659	116
	35	7	0.597	105
	36	7	0.642	113
	49	14	0.539	94.6
	50	14	0.535	93.9
	59	21	0.608	107
	60	21	0.580	102
	69	28	0.639	112
	70	28	0.617	108
	79	35	0.646	113
	80	35	0.644	113
	89	42	0.575	101
	90	42	0.576	101
	99	47	0.545	95.6
	100	47	0.543	95.3

¹ The limit of quantitation (LOQ) was 0.0458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

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Table 5 (continued)

Measured Concentrations of PFOS in Freshwater Samples from a
Fathead Minnow Early Life-Stage Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-108-)	Sampling Time (Day)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
1.1	9	0	1.14	104
	10	0	1.13	103
	23	4	1.21	110
	24	4	1.25	114
	37	7	1.13	103
	38	7	1.23	112
	51	14	1.03	93.6
	52	14	1.10	100
	61	21	1.19	108
	62	21	1.24	113
	71	28	1.30	118
	72	28	1.31	119
	81	35	1.30	118
	82	35	1.31	119
	91	42	1.14	104
	92	42	1.19	108
	101	47	1.13	103
	102	47	1.09	99.1
2.3	11	0	2.21	96.1
	12	0	2.27	98.7
	25	4	2.52	110
	26	4	2.46	107
	39	7	2.43	106
	40	7	2.38	103
4.6	13	0	4.56	99.1
	14	0	4.40	95.7
	27	4	4.79	104
	28	4	4.79	104
	41	7	4.46	97.0
	42	7	4.76	103

¹ The limit of quantitation (LOQ) was 0.0458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000915 mg a.i./L) and the dilution factor of the matrix blank samples (50).

001035

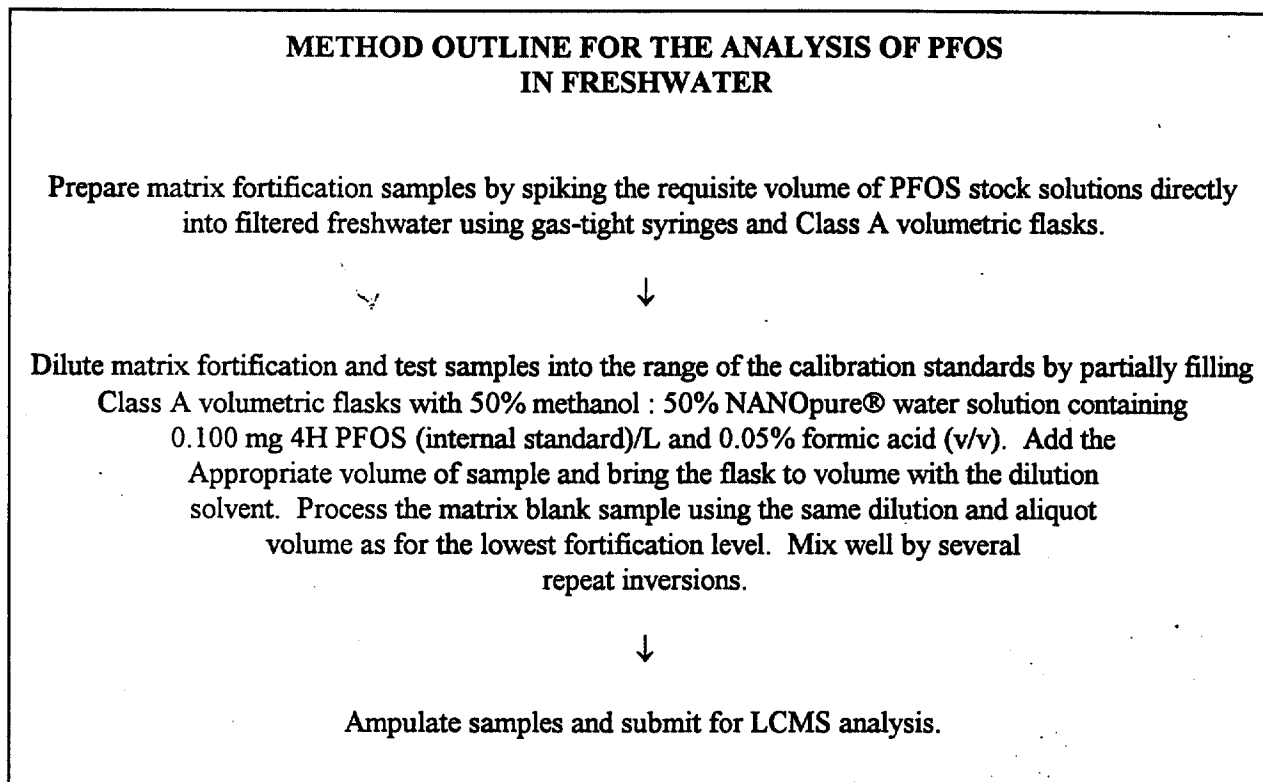


Figure 1. Analytical method flowchart for the analysis of PFOS in freshwater.

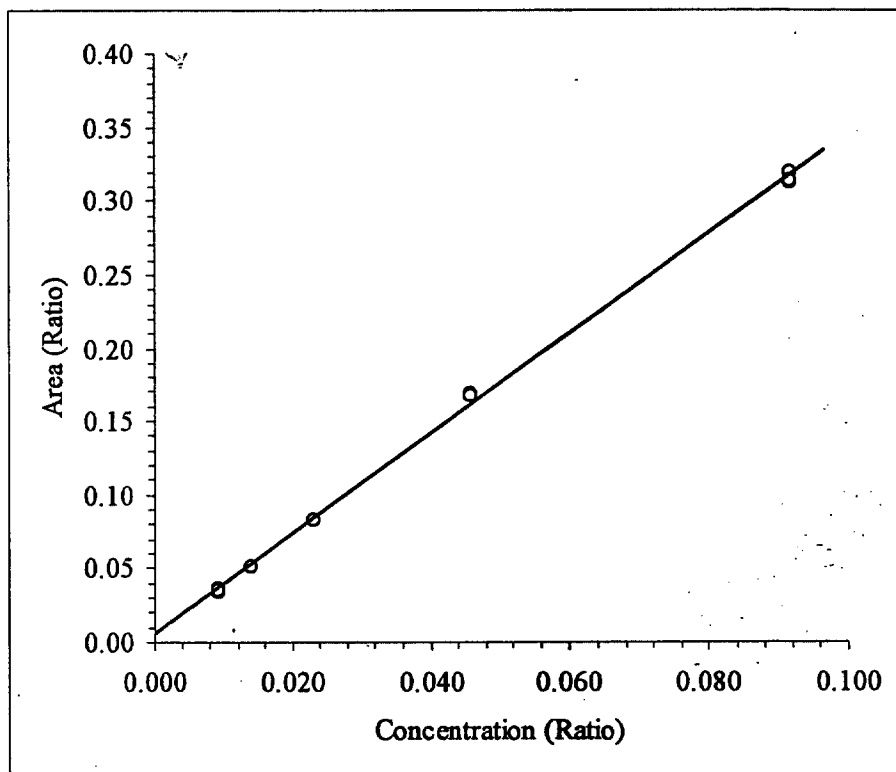


Figure 2. A typical calibration curve for PFOS. Slope = 3.44236; Intercept = 0.00427; $r = 0.9994$. Curve is weighted ($1/x$).

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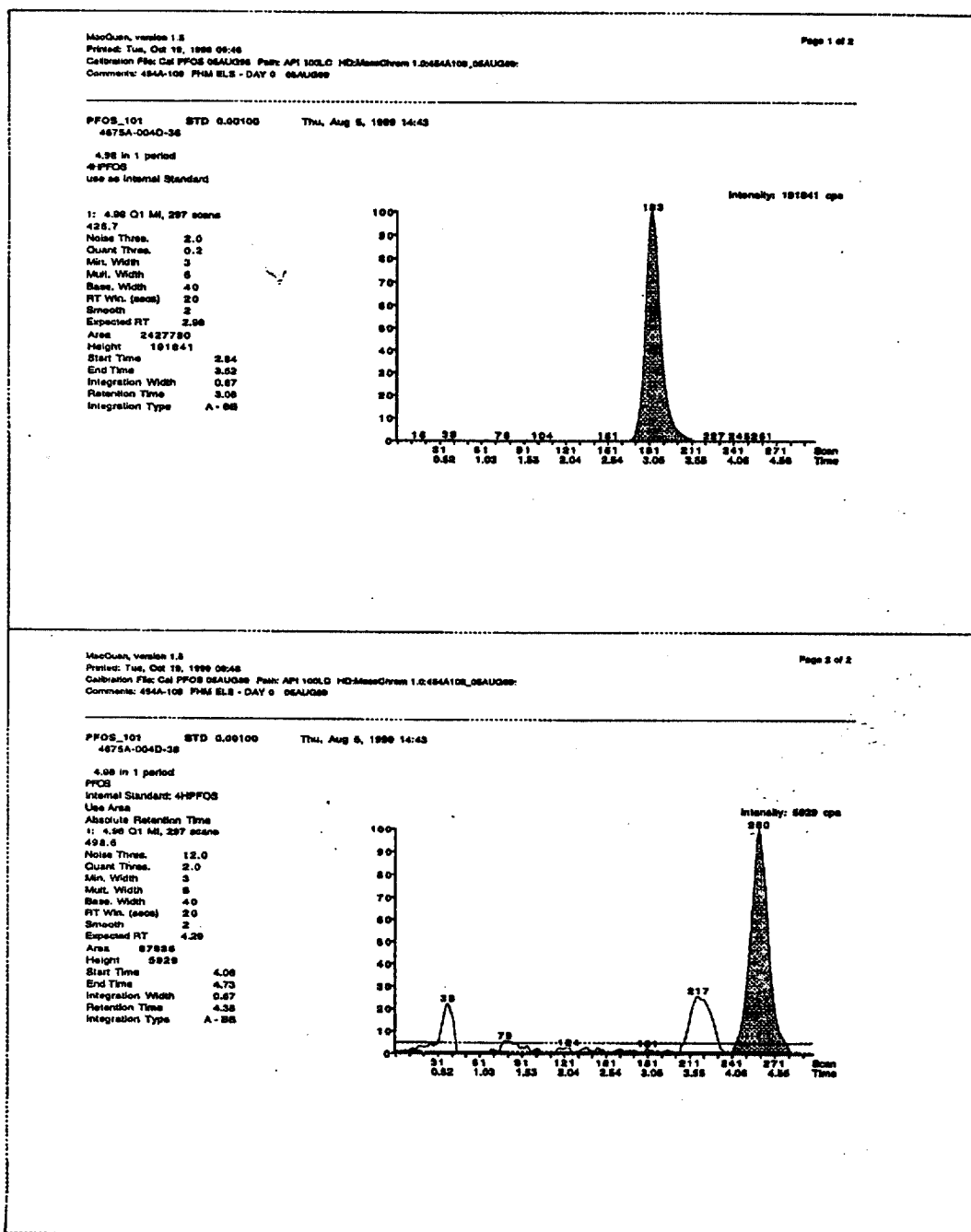


Figure 3. A representative ion chromatogram of a low-level (0.000915 mg a.i./L) PFOS standard.

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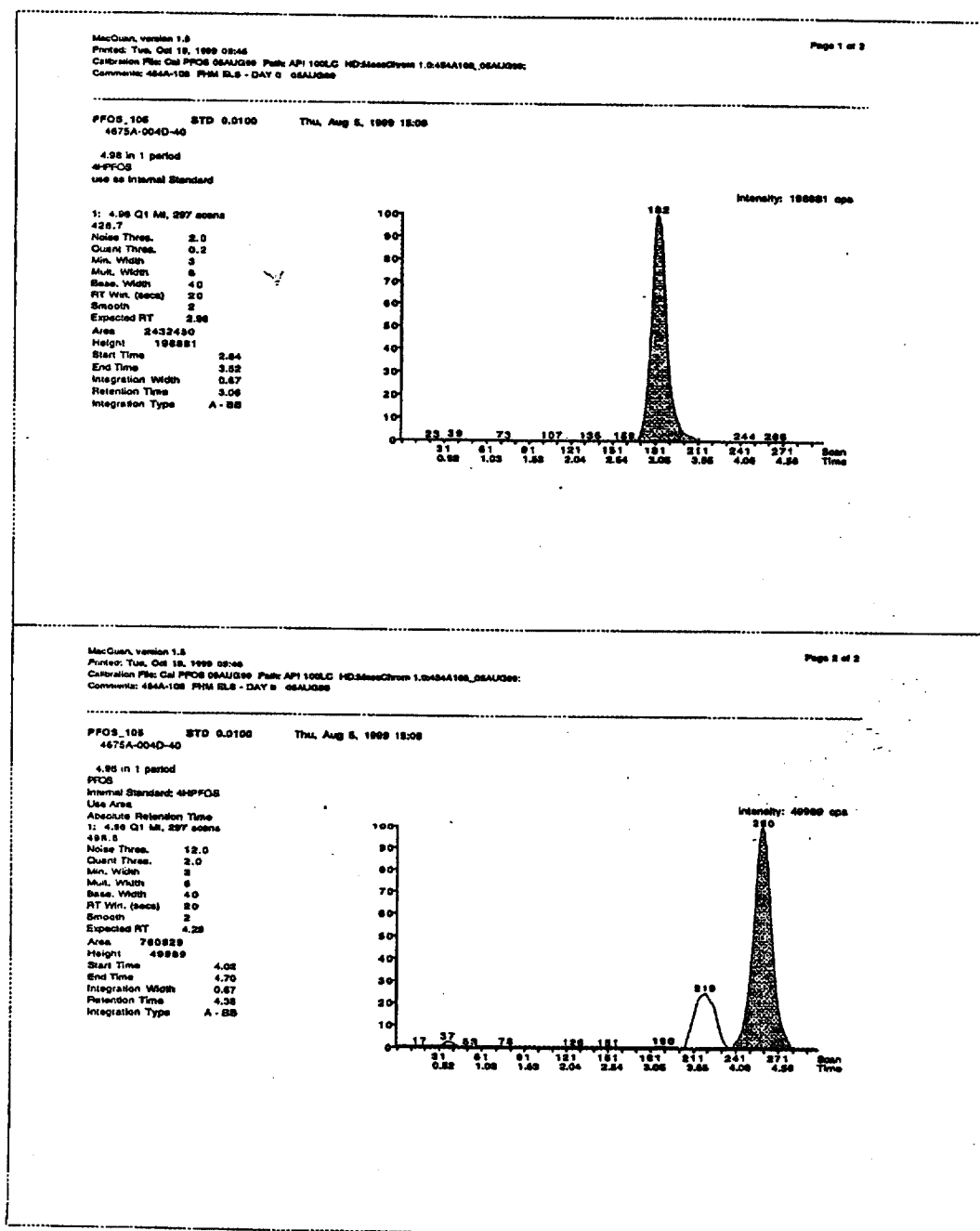


Figure 4. A representative ion chromatogram of a high-level (0.00915 mg a.i./L) PFOS standard.

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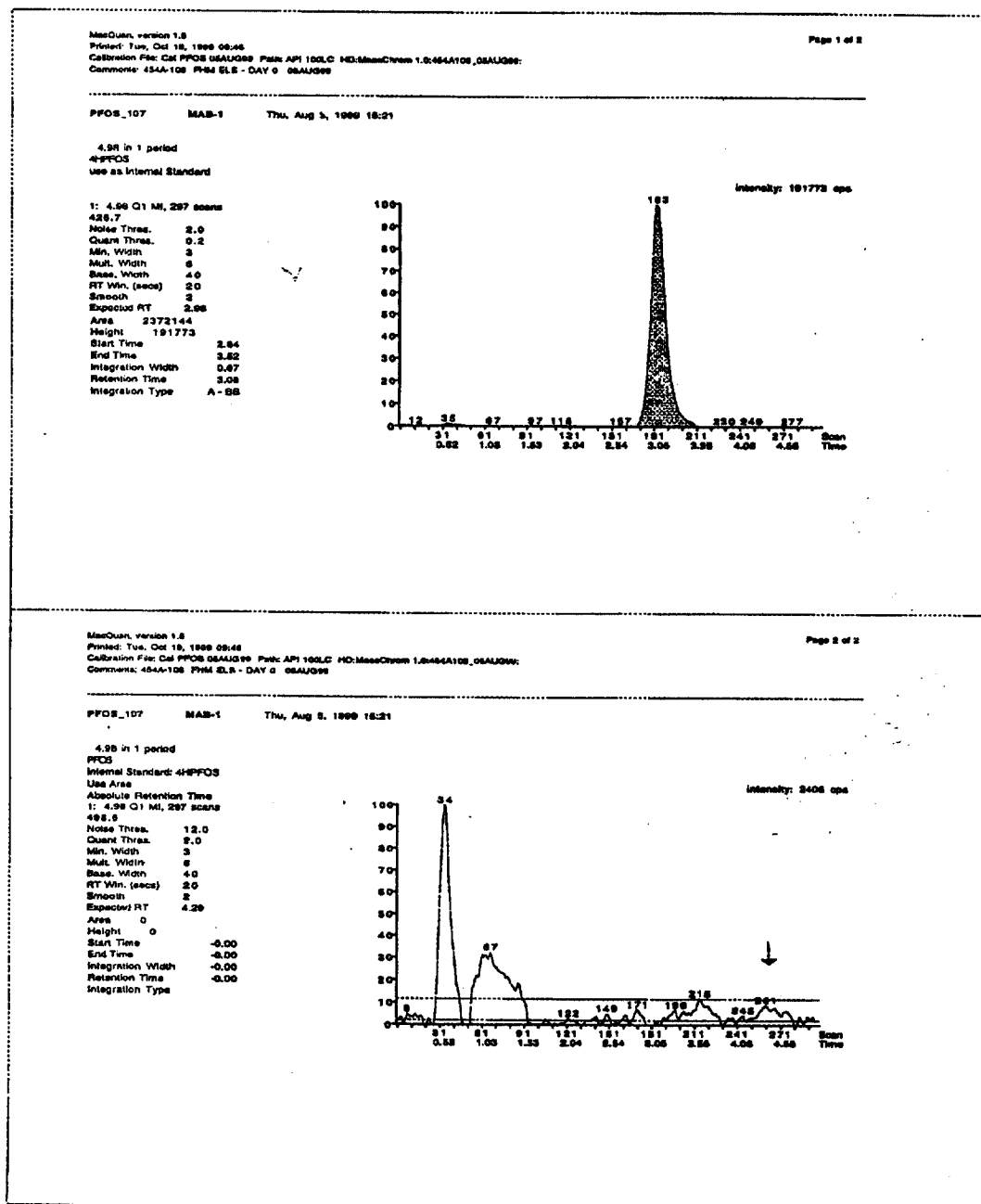


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-108-MAB-1). The arrow indicates the retention time of PFOS.

C01040

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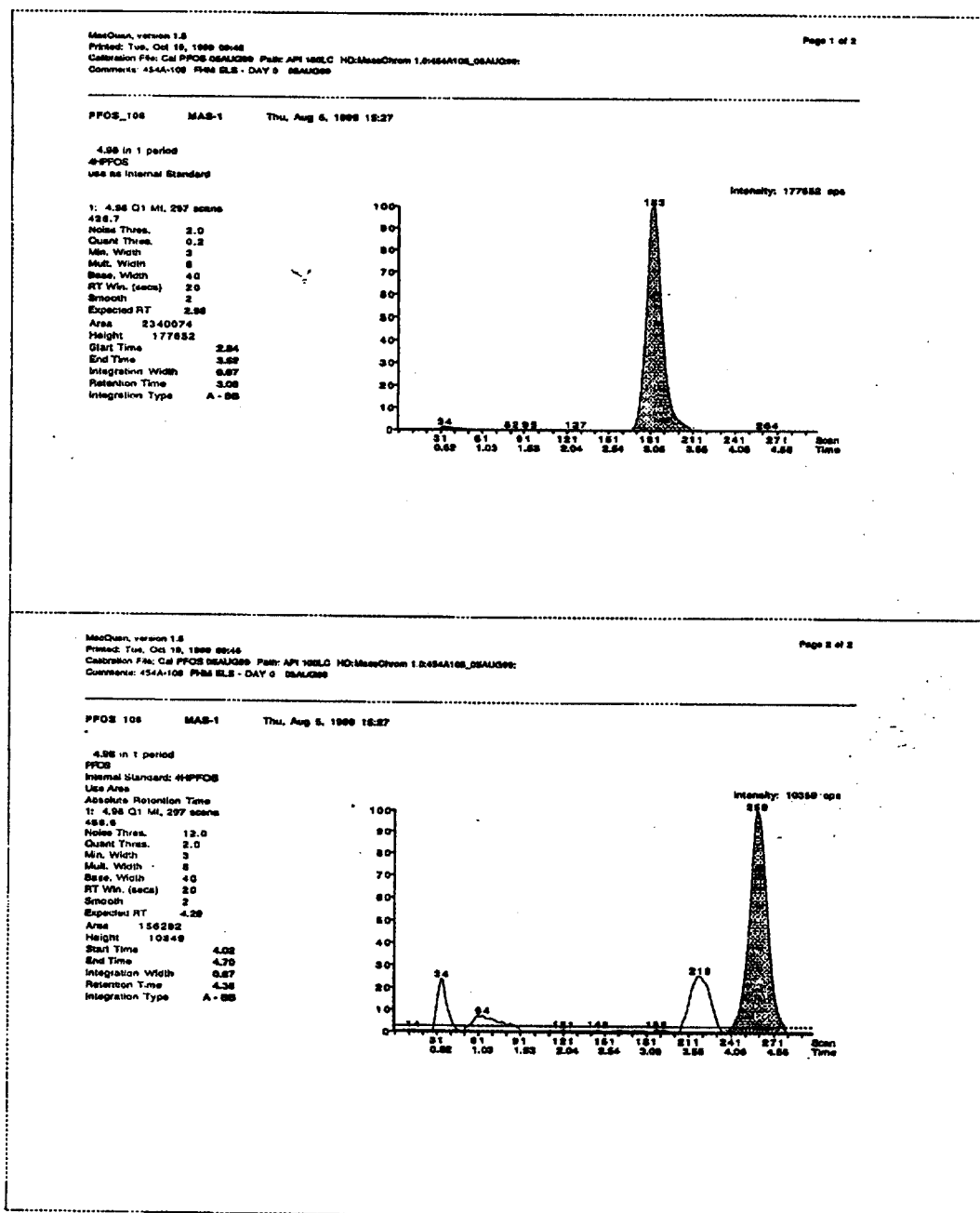


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-108-MAS-1).

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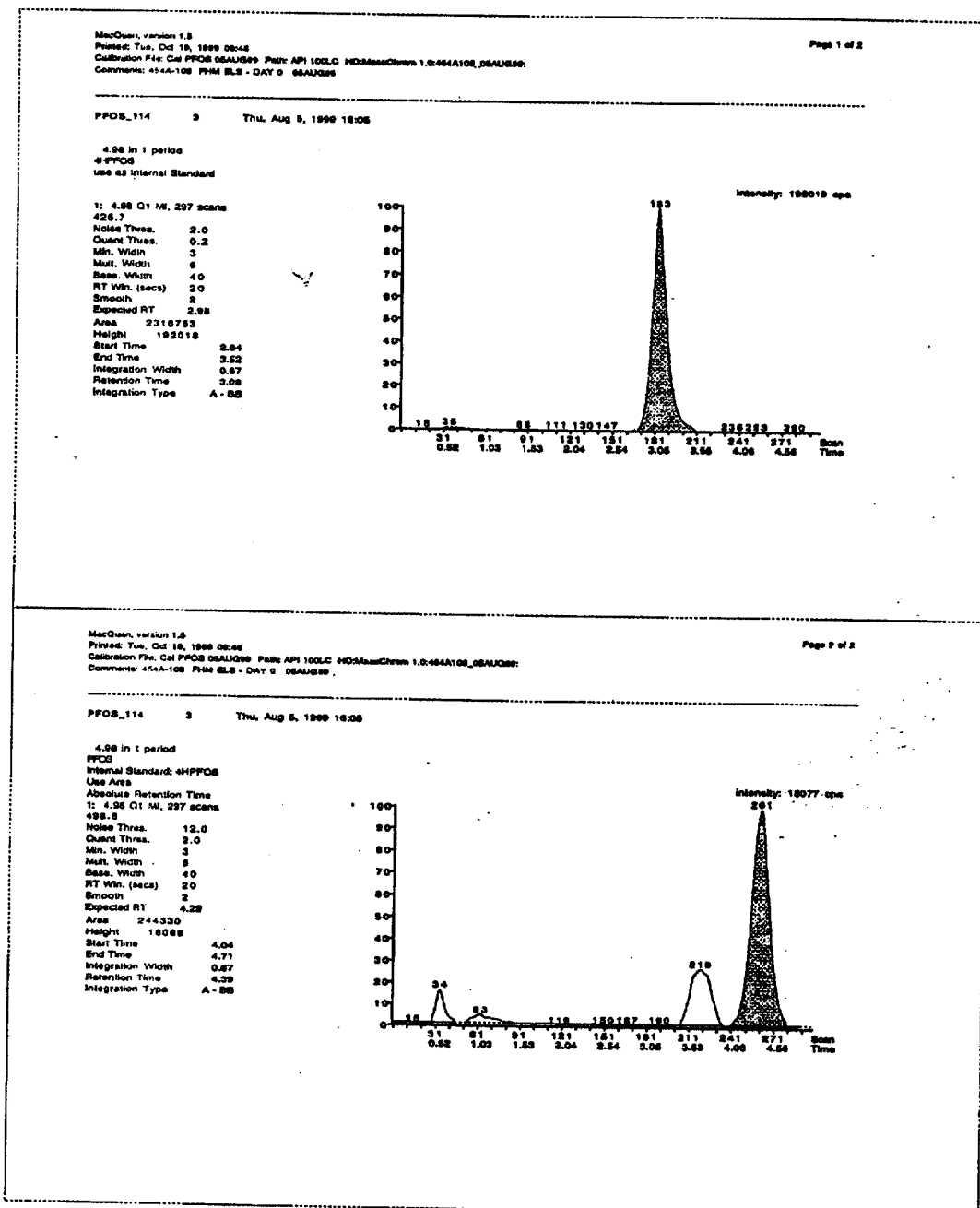


Figure 7. A representative ion chromatogram of a test sample (454A-108-3).

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APPENDIX IV Cumulative Mortality and Treatment-Related Effects¹

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Mean Measured Test Concentration (mg a.i./L)	Replicate	# Hatched	Day 5		Day 6		Day 7		Day 8		Day 9	
			# Dead	Effects	#Dead	Effects	# Dead	Effects	#Dead	Effects	#Dead	Effects
Negative Control	A	18	0	18AN	0	AN	1	AN	1	AN	1	AN
	B	18	1	17AN	0	AN	2	AN	2	AN	2	AN
	C	20	0	18AN	0	AN	0	AN	1	AN	1	AN
	D	18	0	18AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	0	18AN	0	AN	1	AN	1	AN	1	AN
	B	19	0	19AN	0	AN	1	AN	1	AN	1	AN
	C	19	0	19AN	0	AN	1	AN	1	AN	1	AN
	D	20	0	20AN	0	AN	1	AN	1	AN	2	AN
0.30	A	17	0	17AN	0	AN	0	AN	1	AN	1	AN
	B	18	0	18AN	0	AN	0	AN	0	AN	0	AN
	C	18	0	18AN	0	AN	1	AN	1	AN	2	AN
	D	19	0	19AN	0	AN	0	AN	0	AN	0	AN
0.60	A	18	0	18AN	0	AN	0	AN	0	AN	1	AN
	B	19	0	19AN	0	AN	0	AN	1	AN	2	AN
	C	20	0	20AN	0	AN	0	AN	0	AN	1	AN
	D	19	0	19AN	0	AN	1	AN	1	AN	1	AN
1.2	A	18	0	18AN	1	AN	5	AN	5	AN	5	AN
	B	19	0	19AN	3	AN	7	AN	7	AN	7	AN
	C	18	0	18AN	2	AN	6	AN	8	AN	8	AN
	D	19	0	19AN	2	AN	9	AN	9	AN	11	AN
2.4	A	18	0	18AN	14	4E	18	-	18	-	18	-
	B	19	0	19AN	18	1E	19	-	19	-	19	-
	C	20	0	18AN, 2C	12	6AN, 2E	20	-	20	-	20	-
	D	18	0	17AN, 1C	17	1E	18	-	18	-	18	-
4.6	A	19	0	19E,C	19	-	19	-	19	-	19	-
	B	17	0	17E,C	17	-	17	-	17	-	17	-
	C	19	0	19E,C	19	-	19	-	19	-	19	-
	D	19	2	17E,C	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

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APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor: 3M Corporation												
Test Substance: PFOS												
Test Organism: Fathead Minnow, <i>Pimephales promelas</i>												
Dilution Water: Well Water												
Mean Measured Test Concentration (mg a.i./L)	Replicate	# Hatched	Day 10		Day 11		Day 12		Day 13		Day 14	
			# Dead	Effects	# Dead	Effects	# Dead	Effects	Mortality	Effects	Mortality	Effects
Negative Control	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	18	2	AN	3	AN	3	AN	3	AN	3	AN
	C	20	1	AN	1	AN	1	AN	1	AN	1	AN
	D	18	0	AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	19	1	AN	1	AN	1	AN	1	AN	1	AN
	C	19	2	AN	2	AN	2	AN	2	AN	2	AN
	D	20	3	AN	3	AN	3	AN	3	AN	3	AN
0.30	A	17	1	AN	2	AN	2	AN	2	AN	2	AN
	B	18	0	AN	0	AN	0	AN	0	AN	0	AN
	C	18	3	AN	3	AN	3	AN	3	AN	3	AN
	D	19	0	AN	0	AN	0	AN	0	AN	0	AN
0.60	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	19	2	AN	2	AN	2	AN	2	AN	2	AN
	C	20	1	AN	1	AN	1	AN	1	AN	1	AN
	D	19	1	AN	1	AN	1	AN	1	AN	1	AN
1.2	A	18	5	AN	6	AN	6	AN	6	AN	6	AN
	B	19	7	AN	8	AN	8	AN	8	AN	8	AN
	C	18	8	AN	8	AN	8	AN	8	AN	8	AN
	D	19	11	AN	12	AN	12	AN	12	AN	12	AN
2.4	A	18	18	--	18	--	18	--	18	--	18	--
	B	19	19	--	19	--	19	--	19	--	19	--
	C	20	20	--	20	--	20	--	20	--	20	--
	D	18	18	--	18	--	18	--	18	--	18	--
4.6	A	19	19	--	19	--	19	--	19	--	19	--
	B	17	17	--	17	--	17	--	17	--	17	--
	C	19	19	--	19	--	19	--	19	--	19	--
	D	19	19	--	19	--	19	--	19	--	19	--

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor: 3M Corporation												
Test Substance: PFOS												
Test Organism: Fathead Minnow, <i>Pimephales promelas</i>												
Dilution Water: Well Water												
Mean Measured Test Concentration (mg a.i./L)	Replicate	Day 15			Day 16		Day 17		Day 18		Day 19	
		# Hatched	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	Mortality	Effects
Negative Control	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	1	AN	1	AN	1	AN	1	AN	2	AN
	D	18	0	AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	19	1	AN	1	AN	1	AN	1	AN	1	AN
	C	19	2	AN	2	AN	2	AN	2	AN	3	AN
	D	20	3	AN	3	AN	3	AN	3	AN	3	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	0	AN	0	AN	0	AN	0	AN	1	AN
	C	18	3	AN	3	AN	3	AN	3	AN	3	AN
	D	19	0	AN	0	AN	0	AN	0	AN	1	AN
0.60	A	18	1	AN	1	AN	1	AN	1	AN	2	AN
	B	19	2	AN	2	AN	2	AN	2	AN	2	AN
	C	20	1	AN	1	AN	1	AN	1	AN	1	AN
	D	19	1	AN	1	AN	1	AN	1	AN	1	AN
1.2	A	18	6	AN	6	AN	6	AN	6	AN	6	AN
	B	19	8	AN	8	AN	8	AN	9	AN	9	AN
	C	18	8	AN	8	AN	8	AN	9	AN	11	AN
	D	19	12	AN	12	AN	12	AN	12	AN	13	AN
2.4	A	18	18	-	18	-	18	-	18	-	18	-
	B	19	19	-	19	-	19	-	19	-	19	-
	C	20	20	-	20	-	20	-	20	-	20	-
	D	18	18	-	18	-	18	-	18	-	18	-
4.6	A	19	19	-	19	-	19	-	19	-	19	-
	B	17	17	-	17	-	17	-	17	-	17	-
	C	19	19	-	19	-	19	-	19	-	19	-
	D	19	19	-	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor:		3M Corporation										
Test Substance:		PFOS										
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>										
Dilution Water:		Well Water										
Mean Measured Test Concentration (mg a.i./L.)	Replicate	# Hatched	Day 20		Day 21		Day 22		Day 23		Day 24	
			# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	2	AN	2	AN	2	AN	2	AN	2	AN
	D	18	0	AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	1	AN	1	AN	2	AN	2	AN	2	AN
	B	19	1	AN	1	AN	1	AN	1	AN	1	AN
	C	19	3	AN	3	AN	3	AN	3	AN	3	AN
	D	20	3	AN	3	AN	4	AN	4	AN	4	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	1	AN	1	AN	1	AN	1	AN	2	AN
	C	18	3	AN	3	AN	3	AN	3	AN	3	AN
	D	19	1	AN	1	AN	1	AN	1	AN	1	AN
0.60	A	18	2	AN	2	AN	2	AN	2	AN	2	AN
	B	19	2	AN	2	AN	3	AN	3	AN	3	AN
	C	20	1	AN	1	AN	1	AN	1	AN	1	AN
	D	19	1	AN	1	AN	1	AN	1	AN	1	AN
1.2	A	18	6	AN	6	AN	6	AN	6	AN	7	AN
	B	19	9	AN	9	AN	9	AN	10	AN	10	AN
	C	18	11	AN	12	AN	12	AN	12	AN	12	AN
	D	19	13	AN	13	AN	13	AN	13	AN	13	AN
2.4	A	18	18	--	18	--	18	--	18	--	18	--
	B	19	19	--	19	--	19	--	19	--	19	--
	C	20	20	--	20	--	20	--	20	--	20	--
	D	18	18	--	18	--	18	--	18	--	18	--
4.6	A	19	19	--	19	--	19	--	19	--	19	--
	B	17	17	--	17	--	17	--	17	--	17	--
	C	19	19	--	19	--	19	--	19	--	19	--
	D	19	19	--	19	--	19	--	19	--	19	--

¹ Observed Effects: A = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor:	3M Corporation											
Test Substance:	PFOS											
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>											
Dilution Water:	Well Water											
Mean Measured Test Concentration (mg a.i./L)	Replicate	Day 25			Day 26		Day 27		Day 28		Day 29	
		# Hatched	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	2	AN	2	AN	2	AN	2	AN	2	AN
	D	18	0	AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	2	AN	2	AN	2	AN	3	AN	3	AN
	B	19	1	AN	2	AN	3	AN	3	AN	3	AN
	C	19	3	AN	3	AN	3	AN	3	AN	3	AN
	D	20	4	AN	4	AN	5	AN	5	AN	5	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	2	AN	2	AN	2	AN	2	AN	2	AN
	C	18	3	AN	3	AN	3	AN	4	AN	4	AN
	D	19	1	AN	1	AN	1	AN	3	AN	3	AN
0.60	A	18	2	AN	2	AN	2	AN	2	AN	2	AN
	B	19	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	1	AN	2	AN	2	AN	2	AN	3	AN
	D	19	2	AN	3	AN	4	AN	4	AN	4	AN
1.2	A	18	9	AN	11	AN, 2E	13	AN	13	AN	15	AN
	B	19	13	AN	15	AN	15	AN	15	AN	16	AN
	C	18	14	AN	16	AN	16	AN	16	AN	16	AN
	D	19	18	1E	19	-	19	-	19	-	19	-
2.4	A	18	18	-	18	-	18	-	18	-	18	-
	B	19	19	-	19	-	19	-	19	-	19	-
	C	20	20	-	20	-	20	-	20	-	20	-
	D	18	18	-	18	-	18	-	18	-	18	-
4.6	A	19	19	-	19	-	19	-	19	-	19	-
	B	17	17	-	17	-	17	-	17	-	17	-
	C	19	19	-	19	-	19	-	19	-	19	-
	D	19	19	-	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor:		3M Corporation										
Test Substance:		PFOS										
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>										
Dilution Water:		Well Water										
Mean Measured Test Concentration (mg a.i./L)	Replicate	Day 30			Day 31		Day 32		Day 33		Day 34	
		# Hatched	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	1	AN	1	AN	1	AN	1	AN	1	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	2	AN	2	AN	2	AN	3	AN	3	AN
	D	18	0	AN	0	AN	0	AN	0	AN	0	AN
0.15	A	18	3	AN	3	AN	3	AN	3	AN	3	AN
	B	19	3	AN	3	AN	3	AN	4	AN	4	AN
	C	19	3	AN	3	AN	3	AN	4	AN	4	AN
	D	20	5	AN	5	AN	5	AN	5	AN	5	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	2	AN	2	AN	2	AN	2	AN	2	AN
	C	18	4	AN	4	AN	4	AN	5	AN	5	AN
	D	19	3	AN	3	AN	3	AN	3	AN	3	AN
0.60	A	18	2	AN	2	AN	3	AN	3	AN	3	AN
	B	19	4	AN	4	AN	4	AN	5	AN	5	AN
	C	20	3	AN	3	AN	4	AN	7	AN	7	AN
	D	19	4	AN	4	AN	4	AN	6	AN	6	AN
1.2	A	18	15	AN	15	AN	15	AN	15	AN	15	AN
	B	19	17	AN	17	AN	17	AN	17	AN	17	AN
	C	18	16	AN	17	AN	17	AN	17	AN	17	AN
	D	19	19	-	19	-	19	-	19	-	19	-
2.4	A	18	18	-	18	-	18	-	18	-	18	-
	B	19	19	-	19	-	19	-	19	-	19	-
	C	20	20	-	20	-	20	-	20	-	20	-
	D	18	18	-	18	-	18	-	18	-	18	-
4.6	A	19	19	-	19	-	19	-	19	-	19	-
	B	17	17	-	17	-	17	-	17	-	17	-
	C	19	19	-	19	-	19	-	19	-	19	-
	D	19	19	-	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor:		3M Corporation										
Test Substance:		PFOS										
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>										
Dilution Water:		Well Water										
Mean Measured Test Concentration (mg a.i./L)	Replicate	Day 35			Day 36		Day 37		Day 38		Day 39	
		# Hatched	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	1	AN	1	AN	2	AN	2	AN	2	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	3	AN	3	AN	3	AN	3	AN	3	AN
	D	18	0	AN	0	AN	1	AN	1	AN	1	AN
0.15	A	18	3	AN	3	AN	3	AN	3	AN	3	AN
	B	19	4	AN	4	AN	4	AN	4	AN	4	AN
	C	19	4	AN	4	AN	4	AN	4	AN	4	AN
	D	20	5	AN	5	AN	5	AN	5	AN	5	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	2	AN	2	AN	2	AN	2	AN	2	AN
	C	18	5	AN	5	AN, 1R	7	AN	7	AN	7	AN
	D	19	3	AN	3	AN	3	AN	3	AN	3	AN
0.60	A	18	3	AN	4	AN	4	AN	4	AN	4	AN
	B	19	5	AN	5	AN	5	AN	5	AN	5	AN
	C	20	7	AN	7	AN	7	AN	7	AN	7	AN
	D	19	6	AN	6	AN	6	AN	6	AN	6	AN
1.2	A	18	15	AN	15	AN	15	AN	15	AN	15	AN
	B	19	17	AN	17	AN	17	AN	17	AN	17	AN
	C	18	17	AN	17	AN	17	AN	17	AN	17	AN
	D	19	19	-	19	-	19	-	19	-	19	-
2.4	A	18	18	-	18	-	18	-	18	-	18	-
	B	19	19	-	19	-	19	-	19	-	19	-
	C	20	20	-	20	-	20	-	20	-	20	-
	D	18	18	-	18	-	18	-	18	-	18	-
4.6	A	19	19	-	19	-	19	-	19	-	19	-
	B	17	17	-	17	-	17	-	17	-	17	-
	C	19	19	-	19	-	19	-	19	-	19	-
	D	19	19	-	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

APPENDIX IV (Continued)
Cumulative Mortality and Treatment-Related Effects¹

Sponsor:		3M Corporation										
Test Substance:		PFOS										
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>										
Dilution Water:		Well Water										
Mean Measured Test Concentration (mg a.i./L)	Replicate	Day 40			Day 41		Day 42		Day 43		Day 44	
		# Hatched	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	3	AN	3	AN	3	AN	3	AN	3	AN
	C	20	3	AN	3	AN	3	AN	3	AN	3	AN
	D	18	1	AN	1	AN	1	AN	1	AN	1	AN
0.15	A	18	3	AN	3	AN	3	AN	3	AN	3	AN
	B	19	4	AN	4	AN	4	AN	4	AN	4	AN
	C	19	4	AN	4	AN	4	AN	4	AN	4	AN
	D	20	5	AN	5	AN	5	AN	5	AN	5	AN
0.30	A	17	2	AN	2	AN	2	AN	2	AN	2	AN
	B	18	2	AN	2	AN	2	AN	2	AN	2	AN
	C	18	7	AN	7	AN	7	AN	7	AN	7	AN
	D	19	3	AN	3	AN	3	AN	3	AN	3	AN
0.60	A	18	5	AN	5	AN	5	AN	5	AN	5	AN
	B	19	5	AN	5	AN	5	AN	5	AN	5	AN
	C	20	7	AN	7	AN	7	AN	7	AN	7	AN
	D	19	6	AN	6	AN	6	AN	6	AN	6	AN
1.2	A	18	15	AN	15	AN	15	AN	15	AN	15	AN
	B	19	17	AN	17	AN	17	AN	17	AN	17	AN
	C	20	17	AN	17	AN	17	AN	17	AN	17	AN
	D	19	19	-	19	-	19	-	19	-	19	-
2.4	A	18	18	-	18	-	18	-	18	-	18	-
	B	19	19	-	19	-	19	-	19	-	19	-
	C	20	20	-	20	-	20	-	20	-	20	-
	D	18	18	-	18	-	18	-	18	-	18	-
4.6	A	19	19	-	19	-	19	-	19	-	19	-
	B	17	17	-	17	-	17	-	17	-	17	-
	C	19	19	-	19	-	19	-	19	-	19	-
	D	19	19	-	19	-	19	-	19	-	19	-

¹ Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

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APPENDIX IV (Continued)
Cumulative Percent Mortality and Treatment-Related Effects¹

Sponsor:		3M Corporation						
Test Substance:		PFOS						
Test Organism:		Fathead Minnow, <i>Pimephales promelas</i>						
Dilution Water:		Well Water						
Mean Measured Test Concentration (mg a.i./L)	Replicate	# Hatched	Day 45		Day 46		Day 47	
			# Dead	Effects	# Dead	Effects	# Dead	Effects
Negative Control	A	18	2	AN	2	AN	2	AN
	B	18	3	AN	3	AN	3	AN
	C	20	3	AN	3	AN	3	AN
	D	18	1	AN	1	AN	1	AN
0.15	A	18	3	AN	3	AN	3	AN
	B	19	4	AN	4	AN	4	AN
	C	19	4	AN	4	AN	4	AN
	D	20	5	AN	5	AN	5	AN
0.30	A	17	2	AN	2	AN	2	AN
	B	18	2	AN	2	AN	2	AN
	C	18	7	AN	7	AN	7	AN
	D	19	3	AN	3	AN	3	AN
0.60	A	18	5	AN	6	AN	6	AN
	B	19	5	AN	5	AN	6	AN
	C	20	7	AN	7	AN	7	AN
	D	19	6	AN	6	AN	7	AN
1.2	A	18	15	AN	15	AN	16	AN
	B	19	17	AN	17	AN	18	AN
	C	18	17	AN	17	AN	17	AN
	D	19	19	--	19	--	19	--
2.4	A	18	18	--	18	--	18	--
	B	19	19	--	19	--	19	--
	C	20	20	--	20	--	20	--
	D	18	18	--	18	--	18	--
4.6	A	19	19	--	19	--	19	--
	B	17	17	--	17	--	17	--
	C	19	19	--	19	--	19	--
	D	19	19	--	19	--	19	--

¹Observed Effects: AN = Appears Normal; C = Lethargy; E = Erratic swimming; R = Lying on bottom.

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APPENDIX V

Fish Total Length (mm)

Negative Control

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	26.0	29.6	31.7	27.8
2	28.6	26.1	25.7	27.6
3	26.5	28.3	28.3	28.2
4	31.0	30.9	27.7	30.4
5	31.1	25.2	27.8	25.1
6	25.0	26.2	23.0	28.3
7	25.5	22.3	28.6	27.0
8	27.1	26.5	26.3	28.2
9	27.2	29.7	26.6	20.6
10	27.1	30.2	25.7	22.9
11	25.3	18.4	28.4	24.6
12	32.4	29.0	15.2	24.5
13	24.6	28.9	20.1	25.6
14	26.8	29.1	23.7	31.2
15	27.2	26.8	28.1	24.5
16	21.4	--	28.2	25.5
17	--	--	22.1	20.7
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	27.1	27.1	25.7	26.0
Overall Mean (±s)	26.5 (± 0.721)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX V (Continued)

Fish Total Length (mm)

0.15 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	29.0	23.9	27.9	26.8
2	26.2	25.0	29.8	25.7
3	27.5	26.3	26.6	29.6
4	19.8	28.0	27.0	26.8
5	29.4	23.1	32.1	25.2
6	28.3	23.3	27.9	25.8
7	32.1	26.9	23.0	20.1
8	27.9	27.4	25.7	26.1
9	18.1	30.7	23.8	27.3
10	23.9	26.1	27.7	29.0
11	28.5	24.9	22.3	27.6
12	29.5	28.1	27.7	25.8
13	23.6	26.1	26.8	29.4
14	27.1	24.0	24.2	24.1
15	27.8	30.4	29.0	30.0
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	26.6	26.3	26.8	26.6
Overall Mean ($\pm$ s)	26.6 ($\pm$ 0.208)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX V (Continued)

Fish Total Length (mm)

0.30 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	25.9	30.8	27.8	27.8
2	27.0	25.2	27.2	25.9
3	19.4	24.0	29.1	26.6
4	26.2	27.6	25.0	25.4
5	28.0	26.1	27.9	33.1
6	25.7	27.8	28.5	27.0
7	28.0	22.9	28.3	28.0
8	19.7	26.2	28.8	29.8
9	29.5	24.7	24.5	28.1
10	25.5	26.3	25.6	25.9
11	28.1	28.2	25.4	27.3
12	30.5	27.8	--	27.9
13	21.0	30.3	--	30.4
14	20.6	25.0	--	23.1
15	27.3	25.1	--	22.4
16	--	24.2	--	28.0
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	25.5	26.4	27.1	27.3
Overall Mean ($\pm$ s)	26.6 ($\pm$ 0.813)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX V (Continued)

Fish Total Length (mm)

0.60 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	27.1	30.2	28.0	25.4
2	22.7	22.8	29.0	24.6
3	24.5	30.2	28.7	21.1
4	24.4	25.3	28.1	27.9
5	22.8	22.7	27.3	30.8
6	27.0	24.7	25.8	25.9
7	27.1	28.9	24.5	28.5
8	29.1	25.0	25.0	27.4
9	28.4	22.0	28.7	25.6
10	27.5	26.9	30.0	23.1
11	23.7	26.9	25.1	23.4
12	31.3	29.1	26.4	32.2
13	--	26.3	25.4	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	26.3	26.2	27.1	26.3
Overall Mean (±s)	26.5 (± 0.399)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX V (Continued)

Fish Total Length (mm)

1.2 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	26.2	26.7	28.7	--
2	23.1	--	--	--
3	--	--	--	--
4	--	--	--	--
5	--	--	--	--
6	--	--	--	--
7	--	--	--	--
8	--	--	--	--
9	--	--	--	--
10	--	--	--	--
11	--	--	--	--
12	--	--	--	--
13	--	--	--	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	24.7	26.7	28.7	--
Overall Mean (±s)	26.7 (± 2.02)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX VI

Fish Wet Weight (mg)

Negative Control

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	148.1	247.9	184.1	172.9
2	200.5	158.2	182.9	201.4
3	148.9	187.6	206.2	164.5
4	205.6	227.9	162.8	225.0
5	231.0	143.0	173.9	142.4
6	129.1	137.5	124.9	209.2
7	130.1	82.4	210.4	165.0
8	151.1	127.8	139.9	173.9
9	153.1	169.0	181.5	78.7
10	154.6	238.7	142.0	104.6
11	132.6	64.5	215.6	98.3
12	275.1	180.1	28.5	124.2
13	135.5	202.2	60.4	124.7
14	147.9	196.3	145.9	286.8
15	147.6	163.8	165.0	49.9
16	89.1	--	182.0	144.0
17	--	--	77.4	56.9
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	161.2	168.5	152.0	148.4
Overall Mean ($\pm$ s)	158 ($\pm$ 9.10)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX VI (Continued)

Fish Wet Weight (mg)

0.15 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	182.5	118.0	200.1	195.5
2	139.7	133.9	242.4	122.9
3	166.6	124.4	160.4	204.7
4	67.6	199.8	148.0	142.4
5	222.8	89.0	290.1	125.3
6	205.9	119.0	167.2	173.0
7	240.0	165.0	86.6	58.4
8	176.8	195.9	128.2	146.6
9	55.8	200.8	76.6	177.3
10	104.0	180.1	181.7	198.7
11	198.2	139.1	70.0	177.5
12	231.8	210.3	184.6	123.4
13	103.0	155.0	186.7	202.1
14	156.8	105.0	135.6	112.5
15	152.3	214.3	199.9	219.3
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	160.3	156.6	163.9	158.6
Overall Mean ($\pm$ s)	160 ($\pm$ 3.10)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX VI (Continued)

Fish Wet Weight (mg)

0.30 mg a.i./L

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Fish Number	Replicate			
	A	B	C	D
1	144.5	208.8	206.7	162.4
2	161.6	181.1	208.4	155.3
3	62.4	135.5	193.2	203.6
4	184.3	192.0	147.1	135.8
5	203.7	165.6	185.6	261.8
6	126.7	211.9	209.1	164.2
7	163.1	123.8	201.7	157.0
8	75.6	180.7	220.1	193.2
9	256.3	142.9	126.5	178.3
10	125.6	175.1	145.4	124.0
11	198.9	199.7	150.9	142.7
12	268.4	202.2	--	151.6
13	83.4	224.6	--	234.7
14	53.7	122.1	--	65.6
15	189.8	106.3	--	93.0
16	--	149.2	--	179.2
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	153.2	170.1	181.3	162.7
Overall Mean ($\pm$ s)	167 ($\pm$ 11.9)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VI (Continued)

Fish Wet Weight (mg)

0.60 mg a.i./L

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Fish Number	Replicate			
	A	B	C	D
1	206.7	223.7	180.5	161.2
2	96.2	122.1	225.4	125.6
3	113.2	221.0	200.6	85.1
4	131.0	160.7	189.0	201.3
5	93.3	103.3	174.9	272.7
6	169.8	141.1	156.7	132.8
7	163.2	205.3	148.2	169.3
8	231.5	130.6	172.5	175.3
9	223.1	84.0	231.4	137.2
10	201.2	152.9	236.0	88.0
11	136.2	159.4	151.8	85.9
12	176.3	212.0	177.3	251.8
13	--	177.1	125.1	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	161.8	161.0	182.3	157.2
Overall Mean ($\pm$ s)	166 ($\pm$ 11.3)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VI (Continued)

Fish Wet Weight (mg)

1.2 mg a.i./L

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Fish Number	Replicate			
	A	B	C	D
1	173.3	179.3	220.6	--
2	133.7	--	--	--
3	--	--	--	--
4	--	--	--	--
5	--	--	--	--
6	--	--	--	--
7	--	--	--	--
8	--	--	--	--
9	--	--	--	--
10	--	--	--	--
11	--	--	--	--
12	--	--	--	--
13	--	--	--	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	153.5	179.3	220.6	--
Overall Mean ($\pm$ s)	185 ($\pm$ 33.8)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VII

Fish Dry Weight (mg)

Negative Control

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>
Dilution Water:	Well Water

Fish Number	Replicate			
	A	B	C	D
1	30.3	51.2	40.8	38.8
2	42.5	32.7	40.5	42.4
3	32.0	39.9	40.3	32.9
4	43.0	45.2	33.6	50.4
5	45.6	28.6	37.5	30.3
6	24.6	26.2	24.9	46.2
7	25.4	15.7	47.6	34.0
8	31.5	25.1	30.2	37.0
9	30.2	36.8	37.7	15.4
10	30.0	45.7	27.6	21.0
11	27.6	11.8	43.6	20.1
12	55.0	39.7	06.5	26.1
13	26.6	37.8	10.3	25.2
14	29.4	43.1	30.8	62.9
15	29.7	30.8	32.7	09.3
16	19.4	--	39.5	29.9
17	--	--	16.8	11.5
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	32.7	34.0	31.8	31.4
Overall Mean ($\pm$ s)	32.5 ($\pm$ 1.20)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VII (Continued)

Fish Dry Weight (mg)

0.15 mg a.i./L

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Fish Number	Replicate			
	A	B	C	D
1	39.0	22.3	43.6	39.4
2	27.5	25.8	55.6	26.2
3	35.1	25.1	33.6	40.5
4	13.9	39.8	27.6	32.3
5	46.7	16.9	61.9	27.2
6	41.5	22.0	34.0	38.7
7	49.3	34.8	16.4	13.5
8	36.1	41.1	26.3	32.1
9	10.9	41.2	14.6	38.9
10	19.4	37.5	35.0	46.7
11	44.3	30.3	13.9	38.3
12	50.3	45.3	38.9	23.0
13	21.9	32.7	38.1	41.2
14	28.4	20.7	26.9	23.3
15	32.2	46.6	43.2	48.4
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	33.1	32.1	34.0	34.0
Overall Mean ($\pm s$)	33.3 (± 0.900)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VII (Continued)

Fish Dry Weight (mg)

0.30 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
Fish Number	Replicate			
	A	B	C	D
1	27.1	41.2	39.9	33.1
2	33.9	37.3	47.8	32.1
3	10.9	26.2	40.1	43.7
4	40.0	39.3	30.5	27.1
5	42.3	31.9	39.0	51.4
6	25.8	42.5	45.2	34.1
7	33.3	24.8	44.1	33.1
8	15.6	35.3	46.7	39.7
9	52.2	28.8	24.4	34.7
10	25.8	34.3	28.2	24.2
11	42.7	40.4	32.5	28.7
12	56.0	39.5	--	32.1
13	18.2	45.8	--	50.1
14	11.7	23.2	--	14.5
15	41.0	20.1	--	19.0
16	--	27.1	--	36.4
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	31.8	33.6	38.0	33.4
Overall Mean ($\pm$ s)	34.2 ($\pm$ 2.70)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VII (Continued)

Fish Dry Weight (mg)

0.60 mg a.i./L

Sponsor: 3M Corporation
 Test Substance: PFOS
 Test Organism: Fathead Minnow, *Pimephales promelas*
 Dilution Water: Well Water

Fish Number	Replicate			
	A	B	C	D
1	43.9	44.7	38.5	28.8
2	18.8	23.1	46.3	23.9
3	23.1	44.4	39.9	15.8
4	25.1	32.7	36.9	40.0
5	18.2	20.6	35.1	54.2
6	33.0	25.3	30.1	24.6
7	31.3	42.1	27.4	34.5
8	48.6	26.9	32.5	35.8
9	49.0	14.3	46.8	27.9
10	42.9	30.4	49.1	17.1
11	27.0	31.7	29.6	16.3
12	59.8	42.4	37.9	51.1
13	--	32.4	24.0	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	35.1	31.6	36.5	30.8
Overall Mean ($\pm$ s)	33.5 ($\pm$ 2.70)			

Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.

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APPENDIX VII (Continued)

Fish Dry Weight (mg)

1.2 mg a.i./L

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Fathead Minnow, <i>Pimephales promelas</i>			
Dilution Water:	Well Water			
	Replicate			
Fish Number	A	B	C	D
1	33.3	34.2	42.6	--
2	25.6	--	--	--
3	--	--	--	--
4	--	--	--	--
5	--	--	--	--
6	--	--	--	--
7	--	--	--	--
8	--	--	--	--
9	--	--	--	--
10	--	--	--	--
11	--	--	--	--
12	--	--	--	--
13	--	--	--	--
14	--	--	--	--
15	--	--	--	--
16	--	--	--	--
17	--	--	--	--
18	--	--	--	--
19	--	--	--	--
20	--	--	--	--
Replicate Mean	29.5	34.2	42.6	--
Overall Mean (±s)	35.4 (± 6.66)			
Note: Total length values were rounded for presentation. Manual calculations of means and standard deviation may differ slightly from those generated using TOXSTAT® 3.5.				

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APPENDIX VIII

Changes to Protocol

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

1. The protocol was amended to add the proposed experimental start and termination dates.
2. The post-hatch growth period was extended to 42 days.
3. Nominal test concentrations were 0.14, 0.29, 0.57, 1.1, 2.3 and 4.6 mg a.i./L.

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APPENDIX IX

Personnel Involved in the Study

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

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Timothy Z. Kendall, Supervisor
4. Kurt R. Drottar, Senior Biologist
5. Jon A. MacGregor, Scientist
6. Mark A. Mank, Laboratory Supervisor

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PROTOCOL

PFOS: AN EARLY LIFE-STAGE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1400

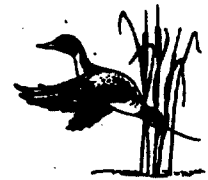
3M Lab Request No. U2723

Submitted to

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



WILDLIFE INTERNATIONAL LTD.



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

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June 10, 1999

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PFOS: AN EARLY LIFE-STAGE TOXICITY TEST WITH THE
FATHEAD MINNOW (*Pimephales promelas*)

SPONSOR:

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

SPONSOR'S REPRESENTATIVE:

Ms. Susan A. Beach

TESTING FACILITY:

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

STUDY DIRECTOR:

Kurt Drottar
Senior Aquatic Biologist

LABORATORY MANAGEMENT:

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

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental

Start Date: _____

Experimental

Termination Date: _____

Project No.: _____

Test Concentrations: Negative Control, 0.16, 0.31, 0.63, 1.3, 2.5 and 5.0 mg a.i./L

Test Substance No.: 4675 Reference Substance No. (if applicable): 4526

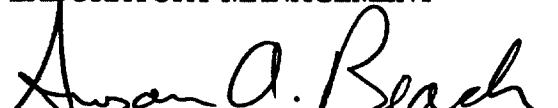
PROTOCOL APPROVAL

STUDY DIRECTOR

DATE

LABORATORY MANAGEMENT

DATE


SPONSOR'S REPRESENTATIVE

6/30/99
DATE

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INTRODUCTION

Wildlife International, Ltd. will conduct an early life-stage toxicity test with fathead minnow (*Pimephales promelas*) for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1400 (1); *ASTM Standard E1241-88 Standard Guide for Conducting Early Life Stage Toxicity Tests with Fish* (2); and *Standard Evaluation Procedure, Fish Early Life-Stage Test* (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

PURPOSE

The purpose of this study is to determine the effects of a test substance on the time to hatch, hatching success, survival, and growth of fathead minnows (*Pimephales promelas*) during early life stage development.

EXPERIMENTAL DESIGN

Fathead minnow embryos will be exposed to a series of six test concentrations and a negative (Dilution water) control. Test concentrations will be chosen by the Study Director in conjunction with the Sponsor based on acute toxicity data. Each test concentration will be 50% of the next higher treatment level.

The test will begin when groups of newly-fertilized embryos are placed in incubation cups and exposed to test solution. Incubation cups, each containing 20 embryos, will be placed in each of four replicate test chambers per treatment (total of 80 embryos per treatment and control). After hatching, larvae will be released from the incubation cups into the larger test chambers where exposure will continue and growth and mortality will be observed over a 28-day period.

Observations of the effects of the test substance on hatching success, growth, and survival will be used to calculate the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). The NOEC and LOEC will be used to calculate the maximum acceptable toxicant concentration (MATC).

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MATERIALS AND METHODS

Test Substance

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

The Sponsor is responsible for all information related to the test substance including the retention of a reserve sample of the lot or batch of the test substance used in this study. The Sponsor also agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism in water. This route of administration was selected because it represents one possible route of exposure to aquatic organisms. The test substance will be mixed directly with dilution water.

Test Organism

Fathead minnows (*Pimephales promelas*) will be used in this test. This species is representative of an important group of organisms and was selected for use in the test based upon past use and ease of handling in the laboratory. Embryos used to start the test will be approximately 2 to 24-hours old and will be obtained from cultures maintained in the laboratory at Wildlife International, Ltd. or a commercial supplier.

Embryos will be removed from spawning substrates using a gentle rolling motion with the index finger or with a clean razor blade and examined under a microscope to select healthy specimens in approximately the same stage of development. Embryos from a minimum of three spawns will be combined and used to initiate testing. If more than 50% of the eggs from a given substrate are fungused or have not been fertilized, no embryos from that substrate will be used in the test.

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Newly-hatched larvae will be fed live brine shrimp nauplii (*Artemia sp.*) 3 times per day during the first 7 days post-hatch. Thereafter, they will be fed live brine shrimp nauplii 3 times daily on weekdays and at least 2 times daily on weekends until the test is terminated. To ensure that the feeding rate per fish remains constant, the amount of food supplied to each replicate test chamber will be adjusted on a weekly basis to account for losses due to mortality. Fish will not be fed for approximately 48 hours prior to the termination of the test to allow the fish to clear their digestive tracts before measurements of weight are made. Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Loading, defined as the total wet weight of fish per liter of test solution, will not exceed 0.5 grams of fish per liter of solution that passes through a chamber in 24 hours. Instantaneous loading will not exceed 5 grams of fish per liter of test solution present in the test chamber at any given time.

Dilution Water

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

Hardness, mg/L as CaCO_3	145
Alkalinity, mg/L as CaCO_3	190
pH	8.1
Specific Conductance, $\mu\text{mhos/cm}$	330

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

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Test Apparatus

A continuous-flow diluter will be used to provide each concentration of the test substance and a negative (dilution water) control. A peristaltic pump will be used to deliver the test substance to mixing chambers where the test substance will be mixed with dilution water. The flow of dilution water into each mixing chamber will be controlled using rotameters. The rotameters will be calibrated prior to test initiation and at approximately weekly intervals thereafter. After mixing, test solutions will be split to each replicate chamber. The proportion of water split to each replicate will be checked prior to the test and at approximately weekly intervals thereafter to ensure that these flow rates vary by no more than $\pm 10\%$ of the mean flow rate of the four replicates.

The diluter will be adjusted so that each test chamber receives at least 5 volume additions of test solution every 24 hours. Peristaltic pumps will be calibrated before each study and at approximately weekly intervals thereafter. The delivery of test substance to test chambers will begin at least 48 hours prior to the test in order to establish equilibrium concentrations of the test substance. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Embryo incubation cups will be constructed from glass cylinders approximately 50 mm in diameter, with 425- μ m nylon or Teflon® screen attached to the bottom using silicone sealant. Test chambers will be 9-L glass aquaria filled with approximately 7 L of water. Test chambers will be positioned in a temperature-controlled environmental chamber to maintain a temperature of $25 \pm 1^\circ\text{C}$. Test chambers will be labelled with project number, replicate and test concentration.

Environmental Conditions

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

The test will be conducted at a target temperature of $25 \pm 2^\circ\text{C}$. Temperature will be monitored and recorded continuously during the entire test in one control replicate using a Fulscope ER/C Recorder (1900 J Series Model A) or equivalent. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation and at least weekly thereafter. The temperature in each test chamber also will be

measured using a liquid-in-glass thermometer at the beginning of the test, at weekly intervals during the test, and at the end of the test.

Dissolved oxygen will be measured in alternate replicates of each treatment and control group at the beginning of the test, daily during the first 7 days of the test, at weekly intervals during the test and at test termination using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, dissolved oxygen measurements will be made daily in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in alternate replicates of each treatment and control group at test initiation, at weekly intervals during the test and at test termination using a Fisher Accumet Model 915 pH meter, or equivalent. If a treatment level reaches 100% mortality, temperature, dissolved oxygen and pH measurements will be taken at that time, then discontinued.

Hardness, alkalinity, and specific conductance will be measured in the dilution water and in the highest treatment level at test initiation, at weekly intervals during the test and at test termination. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (4). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

Procedures

Prior to test initiation, embryo incubation cups will be impartially placed in glass beakers containing dilution water within 3°C of the test temperature. In order to control bias, groups of one, two or three embryos will be indiscriminately distributed among the cups until each contains 20 embryos. No other potential sources of bias are expected to affect the results of the study. Two incubation cups will be placed in each duplicate test chamber to achieve a total of 40 embryos per replicate and 80 embryos per treatment. Incubation cups will be attached to a rocker arm and suspended in the water column of the test chambers. The reciprocating motion of the rocker arm (approximately 2 rpm) will facilitate circulation of test solution around the embryos during the incubation period.

During the first day of exposure, embryos will be observed twice for mortality and eggs with fungus. Thereafter, until hatching is complete, embryo mortality and the removal of dead embryos and/or embryos with fungus will be performed once daily. After hatch, larvae will be counted and released into the test chamber. The test will be repeated if the percentage of embryos in the control group that hatches successfully is less than 66%, or if the post-hatch survival in any control chamber is less than 70%.

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When hatching has reached >90% in the control group, larvae will be released to their respective test chambers. Any unhatched embryos will be kept in the egg cups until they have hatched or until death of the embryo occurs. After the initial release of the larvae, the exposure will continue for at least 28 days.

Biological Measurements

Data on time to hatch, hatching success, survival, and growth (wet weight, dry weight and total length) will be collected during the test. Due to high natural mortality that arises from fungal infections during embryonic development, all fungused embryos that are removed during the first 24 hours of incubation will be considered natural mortality (D.A. Benoit, personal communication). Daily observations of fry mortality, unusual behavior, and overall appearance will be made during the 28-day post hatch growth period. At test termination, all surviving fish will be retained for measurements of wet weight, dry weight, and total length.

Sampling for Analytical Measurements

Prior to exposure water samples will be collected from two replicate test chambers of each treatment group and the negative control. Water samples also will be collected from two alternating replicate test chambers at the beginning of the test, on Day 4 of the test, at approximately weekly intervals during the test, and at the end of the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Pretest ^a	Day 0	Day 4	Day 7	Day 14	Day 21	Day 28	End
Control	2	2	2	2	2	2	2	2
Level 1-Low Concentration	2	2	2	2	2	2	2	2
Level 2	2	2	2	2	2	2	2	2
Level 3	2	2	2	2	2	2	2	2
Level 4	2	2	2	2	2	2	2	2
Level 5	2	2	2	2	2	2	2	2
Level 6-High Concentration	2	2	2	2	2	2	2	2
Totals	14	14	14	14	14	14	14	14

^aPre-test samples will be collected after conditioning of the diluter. More than one pre-test sampling interval may be required, depending upon the Sponsors needs, and additional sampling and results will be documented in the raw data and included in the final report.

Total Number of Verification Samples = 112

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The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, water samples also will be collected from at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

Analytical Measurements

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

Data Analyses

The endpoints used to determine the statistical significance of toxicant-related effects on fathead minnows include hatching success, survival of juvenile fish, total length, wet weight and dry weight of surviving fish. The data sets will be evaluated for normality and homogeneity of variances. Data transformations will be employed, if necessary, to correct the data for non-normality or heterogeneous variances.

When the data meet the conditions of normality and homogeneity of variances, tests for significant differences will use 2 X 2 contingency tables and analysis of variance (ANOVA) procedure with an appropriate means test (e.g., Dunnett's test, Duncan's test, Tukey's test, etc.). When the conditions of normality and homogeneity of variances are not met, and no transformations are successful in correcting the condition, then nonparametric statistical procedures will be used. The results of the statistical analyses will aid in the determination of the NOEC and LOEC. The maximum acceptable toxicant concentration will be calculated as the geometric mean of the NOEC and LOEC.

RECORDS TO BE MAINTAINED

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

1. A copy of the signed protocol.

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2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. History of the test organism.
5. Weight and length measurements.
6. Results of rangefinding tests, when applicable.
7. Stock solution calculation and preparation.
8. Observations made during the test.
9. Water chemistry calculations (e.g., hardness and alkalinity).
10. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.
11. Statistical calculations.
12. Test conditions and physical/chemical measurements.
13. Calculation and preparation of test concentrations.
14. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed. It is the responsibility of the Sponsor to provide the final date that data are recorded for chemistry pathology and/or supporting evaluations that may be generated at other laboratories.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures as stated in the approved protocol, including any changes in the original protocol.
5. The test substance identification, including name, chemical abstract number or code number, strength, purity, composition, and other characteristics provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.

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8. A description of the test organisms, including the source of the test organisms, scientific name, age, life stage, means and ranges of weights and lengths, observed diseases and treatments.
9. A description of the preparation of the test solutions, the methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
10. A description of circumstances that may have affected the quality or integrity of the data.
11. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
12. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
13. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
14. Statistical methods employed for analyzing the data.
15. The location where raw data and final report are to be stored.
16. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
17. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the alteration. Amendments will be signed and dated by the Study Director.

CHANGING OF PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17);

and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

- 1 **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1400: *Fish Early-Life Stage Toxicity Test*.
- 2 **ASTM Standard E1241-92.** 1977. *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes*. American Society for Testing and Materials.
- 3 **United States Environmental Protection Agency.** 1986. *Standard Evaluation Procedure, Fish Early Life-Stage Test*. Office of Pesticide Programs, Hazard Evaluation Division. EPA 540/9-86-138.
- 4 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

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APPENDIX I

IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

- I. Test Substance Identity (name to be used in the report): PFOS (Perfluorooctane Sulfonic Acid Potassium Salt
Reference Standard (if applicable): Analytical Standard: N/A

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

Test Substance Sample Code or Batch Number: Lot 217

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

II. Test Substance Characterization

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

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International, Ltd.

Ambient

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

Other pertinent stability information:

IV. Test Concentrations:

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

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

V. Toxicity Information:

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

Aquatic:

Invertebrate Toxicity (EC/LC50)

Fish Toxicity (LC50)

Daphnia magna: 27 mg/L

Rainbow Trout: 11 mg/L

Daphnia magna: 50 mg/L

Fathead Minnow: 38 mg/L

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

Please see MSDS

001082

APPENDIX II

Analytical Method Provided by Sponsor

Samples will be analyzed based upon procedures provided by the Sponsor in the following analytical methods:

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

A copy of the above method will be maintained in the raw data. The actual methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

001083

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: AN EARLY LIFE-STAGE TOXICITY TEST WITH THE
FATHEAD MINNOW (*Pimephales promelas*)

PROTOCOL NO.: 454/061099/FAT-ELS/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-108

EFFECTIVE DATE: August 2, 1999

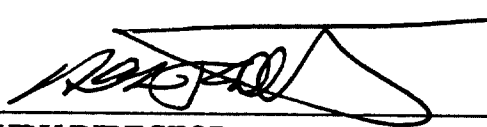
AMENDMENT: Page 2

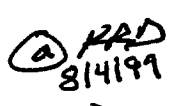
Add: Proposed Dates:

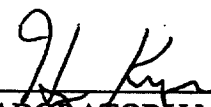
Experimental Start Date: 8/5/99

Experimental Termination Date: 9/6/99

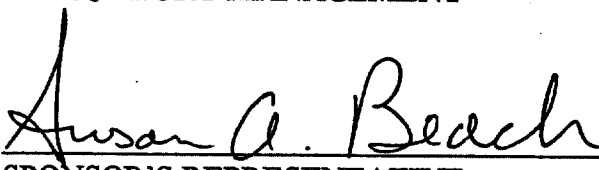
REASON: The above information was not known when the protocol was signed by the Study Director.


STUDY DIRECTOR


~~8/4~~ 8/4/99
DATE


LABORATORY MANAGEMENT

8/4/99
DATE


SPONSOR'S REPRESENTATIVE

8/12/99
DATE

001084