PFOS:
A 96-HOUR TOXICITY TEST WITH THE FRESHWATER DIATOM (*Navicula pelliculosa*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-112

3M LAB REQUEST NO. U2723

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.5400

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STUDY INITIATION DATE: January 28, 2000

STUDY COMPLETION DATE: March 26, 2001

Submitted to

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Wildlife International, Ltd.

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-112

STUDY COMPLETION: March 26, 2001

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 prior to its use in the study; 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 has been recharacterized in accordance with GLP (September 7, 2000).

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

STUDY DIRECTOR:

Cary A. Sutherland
Laboratory Supervisor

3/26/01
DATE

SPONSOR:

Sponsor

5/15/01
DATE

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:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	February 24, 2000	February 24, 2000	February 29, 2000
Analytical Standard Preparation	February 25, 2000	February 25, 2000	March 7, 2000
Recovery Phase Cell Counts	March 7, 2000	March 9, 2000	March 14, 2000
Analytical Data and Draft Report	May 12 and 15, 2000	May 16, 2000	May 16, 2000
Biological Data and Draft Report	June 5 – 8, 2000	June 8, 2000	June 23, 2000
Analytical Report, Second Draft	March 12, 2001	March 12, 2001	March 16, 2001
Biological Report, Second Draft	March 14 –15, 2001	March 15, 2001	March 20, 2001
Final Report	March 21, 2001	March 21, 2001	March 26, 2001

James H. Coleman

James H. Coleman
Quality Assurance Representative

3-26-01

DATE

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-112

STUDY DIRECTOR:

Cary A. Sutherland
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3/26/01
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DATE

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Rochelle R. Robideau
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, MD 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	454A-112
TEST SUBSTANCE:	PFOS (Perfluorooctanesulfonate, Potassium Salt) IUPAC Name: 1-Octanesulfonic acid,1,1,2,2,3,3,4,4,5,5,6,6, 7,7,8,8,8-heptadecafluoro-potassium salt; CAS #2795-39-3
STUDY:	PFOS: A 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*)
NOMINAL TEST CONCENTRATIONS:	Negative Control, 61.5, 81.3, 110, 147, 198, 264 and 347 mg a.i./L
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L
TEST DATES:	Experimental Start (OECD) – February 24, 2000 Experimental Start (EPA) – February 25, 2000 Exposure Termination – February 29, 2000 Experimental Termination – March 7, 2000
LENGTH OF EXPOSURE:	96 Hours

TEST ORGANISM:	Freshwater Diatom (*Navicula pelliculosa*)
SOURCE OF TEST ORGANISMS:	Wildlife International, Ltd. Easton, Maryland 21601

SUMMARY (Continued)

CELL DENSITY:	
72-HOUR EC50:	242 mg a.i./L
95% CONFIDENCE LIMITS:	200 - 276 mg a.i./L
96-HOUR EC10:	<62.3 mg a.i./L
95% CONFIDENCE LIMITS:	Not Calculable
96-HOUR EC50:	263 mg a.i./L
95% CONFIDENCE LIMITS:	217 - 299 mg a.i./L
96-HOUR EC90:	322 mg a.i./L
95% CONFIDENCE LIMITS:	310 - 328 mg a.i./L
72-HOUR NOAEC:	<62.3 mg a.i./L
96-HOUR NOAEC:	150 mg a.i./L

AREA UNDER THE GROWTH CURVE:	
72-HOUR EC50:	
95% CONFIDENCE LIMITS:	246 mg a.i./L 210 - 277 mg a.i./L
96-HOUR EC10:	
95% CONFIDENCE LIMITS:	<62.3 mg a.i./L Not Calculable
96-HOUR EC50:	252 mg a.i./L
95% CONFIDENCE LIMITS:	220 - 285 mg a.i./L
96-HOUR EC90:	319 mg a.i./L
95% CONFIDENCE LIMITS:	308 - 326 mg a.i./L
72-HOUR NOAEC:	<62.3 mg a.i./L
96-HOUR NOAEC:	<62.3 mg a.i./L

GROWTH RATE:	
72-HOUR EC50:	295 mg a.i./L
95% CONFIDENCE LIMITS:	288 - 305 mg a.i./L
96-HOUR EC10:	243 mg a.i./L
95% CONFIDENCE LIMITS:	209 - 295 mg a.i./L
96-HOUR EC50:	305 mg a.i./L
95% CONFIDENCE LIMITS:	295 - 316 mg a.i./L
96-HOUR EC90:	>335 mg a.i./L
95% CONFIDENCE LIMITS:	Not Calculable
72-HOUR NOAEC:	206 mg a.i./L
96-HOUR NOAEC:	206 mg a.i./L

All values are mean measured test concentrations.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for 3M Corporation at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from February 25, 2000 to February 29, 2000, with the recovery phase completed on March 7, 2000. Raw data generated by Wildlife International, Ltd and a copy of the final report are filed under Project Number 454A-112 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the toxicity of PFOS (Perfluorooctanesulfonate, Potassium Salt) to the growth of the freshwater diatom, *Navicula pelliculosa*, during a 96-hour exposure period.

EXPERIMENTAL DESIGN

The freshwater diatom, *Navicula pelliculosa*, was exposed to a geometric series of seven test concentrations and a negative (culture medium) control under static conditions for 96 hours. Three replicate test chambers were maintained for each treatment and control group. One additional replicate for each treatment and control group was maintained for analytical sampling at 72 hours. In addition, two "abiotic" replicates (test solution without algae) were prepared for the highest test concentration. Nominal test concentrations were selected in consultation with the Sponsor and were based upon the results of range finding tests. The nominal test concentrations were 61.5, 81.3, 110, 147, 198, 264 and 347 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test medium collected from each treatment and the control group at test initiation, at approximately 72 hours, and at test termination.

At test initiation, an inoculum of the algal cells was prepared at a concentration of approximately 1.0×10^6 cells/mL. The concentration of algal cells in the inoculum was verified and 1.0 mL was added to each test chamber to achieve a nominal concentration of approximately 1.0×10^4 cells/mL. Samples were collected from each replicate test chamber at approximately 24-hour intervals during the test to determine

cell densities. Cell densities were measured for each replicate and were used to calculate areas under the growth curve and growth rates. Percent inhibition values relative to the control were calculated for each parameter over the 96 hour exposure period. EC50 values based upon cell densities, areas under the growth curve and growth rates were calculated for each 24 hour interval. EC10 and EC90 values were calculated, if possible, for the 72 and 96-hour intervals. The no-observed-adverse-effect-concentration (NOAEC) was determined based upon statistical evaluation of the 72-hour and 96-hour results. At the end of the 96-hour exposure, algistatic effects were differentiated from algicidal effects.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*)". The protocol was based on procedures outlined in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II* (draft)(1).

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 98.9% and an expiration date of 2008. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated September 7, 2000 indicated a purity of 86.9% and an expiration date of August 31, 2001. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 61.5, 81.3, 110, 147, 198, 264 and 347 mg a.i./L, based on a test substance purity of 86.9%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or were Teflon®-lined. Individual test solutions were prepared in algal medium at each of the seven nominal concentrations. The test solutions were stirred with a magnetic stir plate for approximately 24 hours to aid in the solubilization of the test substance. All test solutions appeared clear and colorless.

Test Organism

The freshwater diatom, *Navicula pelliculosa*, was selected as the test species for this study. The species is representative of an important group of freshwater algae, and was selected for use in the test based upon a past history of use and ease of culturing in the laboratory. Original algal cultures were obtained from UTEX - The Culture Collection of Algae at the University of Texas at Austin and have been maintained in culture medium at Wildlife International, Ltd., Easton, Maryland. Algal cells used in this test were obtained from Wildlife International, Ltd. cultures that had been actively growing in culture medium for at least two weeks prior to test initiation. The negative control organisms were expected to exhibit exponential growth over the 96-hour exposure period. Exponential growth, defined as the period of growth where the algal cells are dividing at a constant rate, is indicated by the linear section of the growth curve (Figure 1).

Culture Medium

The algal cells were cultured and tested in freshwater algal medium with silica and selenium (2). Stock nutrient solutions were prepared by adding reagent-grade chemicals to Wildlife International, Ltd. well water purified by reverse osmosis. The test medium was prepared by adding appropriate volumes of the stock nutrient solutions to purified well water (Appendix I). The pH of the medium was adjusted to 7.5 ± 0.1 using 0.1 N NaOH and 10% HCl, and the medium was sterilized by filtration (0.22 μm) prior to use. Analyses were performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. The results of the most recent GLP analyses performed to measure the concentrations of selected contaminants in the well water are presented in Appendix II.

Test Apparatus

Test chambers were sterile, 250-mL plastic Erlenmeyer flasks plugged with foam stoppers, and contained 100 mL of test or control medium. The test chambers were labeled with the project number, concentration and replicate, and were indiscriminately positioned daily on mechanical shaker tables in an environmental chamber designed to maintain the desired test temperature throughout the test. The test chambers were shaken continuously at approximately 100 rpm.

Environmental Conditions

Test flasks were held in an environmental chamber at a temperature of 24 ± 2°C. The temperature of a container of water adjacent to the test flasks in the environmental chamber was recorded twice daily during the test using a liquid-in-glass thermometer.

The algae were held under continuous cool-white fluorescent lighting throughout the test. The target light intensity was 4300 ± 10% lux. Light intensity was measured at the four corners and the middle of each shaker table at test initiation using a SPER Scientific Model 840006 light meter.

The pH of the medium prepared for each treatment and control group was measured at test initiation and termination using a Fisher Accumet Model 915 pH meter. Samples for pH measurement at test initiation were collected from the individual batches of test solution prepared for each treatment and control group. At test termination, samples of test solution were collected from pooled replicates of the treatment and control groups for pH measurement.

Algal Growth Measurements

Test medium samples were collected from the treatment and control groups for the determination of algal cell densities. Single samples were collected from each of the three "biological" replicates per treatment and control group at 24-hour intervals during the 96-hour exposure, and were held for a maximum of four days under refrigerated conditions sufficient to inhibit growth until cell counts could be performed. Cell counts were conducted using a hemacytometer and microscope. Each sample was diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. A small amount of each

sample was loaded onto a hemacytometer and 10 grids were counted. The mean number of cells per grid was estimated and this value was used to calculate the cell density of the sample. Using this technique, the minimum quantifiable cell density was 1.0×10^3 cells/mL.

Samples of test solution were collected from each replicate per treatment and control group at the end of the test. These samples were pooled within their respective treatment, and subsamples were removed and examined microscopically for atypical cell morphology (e.g., changes in cell shape or color). Growth of cells in the replicate test chambers also was assessed for aggregations (clumping) of cells and adherence of the cells to the test chamber.

Statistical Analyses

Cell densities, areas under the growth curve, growth rates and percent inhibition were calculated using "The SAS System for Windows", Release 6.12 (3). Area under the growth curve was calculated for the control and treatment groups using the following formula:

$$A = ((N_1-N_o)/2)(t_1)+((N_1+N_2-2N_o)/2)(t_2-t_1)+\ldots+((N_{n-1}+N_n-2N_o)/2)(t_n-t_{n-1})$$

where: A = Area
N_o = Mean nominal number of cells/mL at t_o
N_1 = Mean measured number of cells/mL at t_1
N_2 = Mean measured number of cells/mL at t_2
N_n = Mean measured number of cells/mL at t_n
t_1 = Time of first measurement after beginning of test (hours)
t_2 = Time of second measurement after beginning of test (hours)
t_n = Time of n^{th} measurement after beginning of test (hours)

Growth rates were calculated for the control and each treatment group using the following formula:

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

where: μ = Average specific growth rate
N_o = Mean nominal number of cells/mL at t_o
N_n = Mean measured number of cells/mL at t_n
t_o = Time of beginning of the test (hours)
t_n = Time of n^{th} measurement after beginning of test (hours)

Percent inhibition was calculated for each treatment group as the percent reduction in cell density, area under the growth curve and growth rate relative to the control replicates. The following formula was used:

$$\text{Percent Inhibition} = \frac{\text{Mean Response}_{\text{Control}} - \text{Mean Response}_{\text{Treatment}}}{\text{Mean Response}_{\text{Control}}} \text{ X } 100$$

Cell densities, areas under the growth curve and growth rates were analyzed statistically to estimate the EC10, EC50 and EC90 values (i.e., the theoretical test concentrations that would produce a 10, 50 or 90% reduction in each parameter, respectively) and 95% confidence limits at 72 and 96 hours. EC50 values were also calculated for the 24 and 48-hour time intervals. The EC values and 95% confidence limits were calculated by linear interpolation with treatment response and exposure concentration data using TOXSTAT Version 3.5 (4). Cell densities, areas under the growth curve and growth rates at 72 and 96 hours were evaluated for normality and homogeneity of variances using the Shapiro-Wilk's test and Levene's test, respectively. The treatment groups were then compared to the control using Dunnett's test. Results of the statistical analyses and evaluation of the concentration-response pattern were used to determine the NOAEC values.

Analytical Chemistry

Samples of test medium were collected from the negative control and each treatment group at test initiation, at approximately 72 hours and at test termination to measure concentrations of the test substance. Samples of test medium collected at test initiation were taken from the individual batches of test solution prepared for each treatment and the control group. Samples collected at 72 hours were collected from the additional "analytical" replicates. Samples collected at test termination were a composite of the remaining biotic replicates for each treatment and the control group. The 335 mg a.i./L abiotic replicates were sampled at 72 and 96 hours to determine the stability of the test substance under the conditions of administration. The samples were placed in plastic centrifuge tubes and were analyzed immediately without storage. The 72 and 96-hour samples were centrifuged approximately 5 minutes at approximately 1500 rpm prior to analysis. Analytical procedures used in the analysis of the samples are presented in Appendix III.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in the test solutions are presented in Table 1 and Appendix III. Nominal concentrations used in this study were 61.5, 81.3, 110, 147, 264 and 347 mg a.i./L. Samples collected at the beginning of the test had measured concentrations that ranged from 96.2 to 106% of nominal. Samples collected at 72 hours and at test termination had recoveries that ranged from 98.5 to 106%, and 94.8 to 101% of nominal, respectively. The abiotic replicates from the 347 mg a.i./L treatment had recoveries of 98.2 and 96.8% of nominal at 72 and 96 hours, respectively, which were comparable to the biotic replicate recoveries. When the values obtained at test initiation, at 72 hours and at test termination were averaged, the mean measured test concentrations were 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L, representing 101, 102, 101, 102, 104, 101 and 96.5% of nominal concentrations, respectively. Mean measured test concentrations were used in the calculation of EC values.

Observations and Measurements

Measurements of temperature, light intensity and pH are presented in Tables 2, 3 and 4, respectively. The temperatures ranged from 23.1 to 24.6°C and were within the range established for the test (24 ± 2°C). The light intensity ranged from 3910 and 4510 lux and was within the desired range for the test (approximately 3870 to 4730 lux). Measurements of pH were 7.5 on Day 0 and ranged from 7.7 to 8.9 at 96 hours. The pH of the abiotic replicate at test termination was 7.7, which was comparable to the biotic replicates at the same concentration.

The effect of PFOS upon *Navicula pelliculosa* was determined by evaluating differences in cell densities, areas under the growth curve and growth rates. Mean values for each parameter were used to calculate growth inhibition for each 24-hour period. Mean cell densities, areas under the growth curve and growth rates, and the corresponding percent inhibition, are presented in Tables 5, 6 and 7, respectively. Cell density, area under the growth curve and growth rate for each individual replicate are presented in Appendices IV, V and VI, respectively. EC values and 95% confidence limits calculated for each 24-hour interval based on cell density, area under the growth curve and growth rate are presented in Tables 8, 9 and 10, respectively.

Changes in cell density indicated that exponential growth occurred in the negative control replicates (Figure 1). After 72 hours of exposure, cell density percent inhibition in the 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L treatment groups was 34, 30, 34, 23, 25, 64 and 99%, respectively. Dunnett's test showed that cell density was significantly reduced in the 62.3, 83.2, 111, 266 and 335 mg a.i./L treatment groups in comparison to the negative control ($p \leq 0.05$). Consequently, the NOAEC for 72-hour cell density was < 62.3 mg a.i./L, the lowest concentration tested. After 96 hours of exposure, cell density percent inhibition in the 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L treatment groups was 13, 13, 13, 9.3, 23, 51 and 99%, respectively. Dunnett's test showed that cell density was significantly reduced in the 206, 266 and 335 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96-hour cell density was 150 mg a.i./L.

After 72 hours of exposure, area under the growth curve percent inhibition in the 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L treatment groups was 28, 27, 29, 20, 23, 62 and 99%, respectively. After 96 hours of exposure, percent inhibition was similar to the inhibition at 72 hours and was 24, 22, 24, 16, 24, 58 and 99%, respectively. Dunnett's test showed that area under the growth curve was significantly reduced ($p \leq 0.05$) in all treatments except the 150 mg a.i./L treatment group at both 72 and 96 hours. Consequently, the NOAEC for 72 and 96-hour area under the growth curve was < 62.3 mg a.i./L, the lowest concentration tested.

After 72 hours of exposure, growth rate percent inhibition in the 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L treatment groups was 8.1, 6.7, 7.8, 5.2, 5.5, 20 and 90%, respectively. Dunnett's test showed that growth rate was significantly reduced in the 266 and 335 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 72-hour growth rate was 206 mg a.i./L. After 96 hours of exposure, growth rate percent inhibition in the 62.3, 83.2, 111, 150, 206, 266 and 335 mg a.i./L treatment groups was 2.5, 2.7, 2.5, 1.7, 4.7, 13 and 79%, respectively. Dunnett's test showed that growth rate was significantly reduced in the 266 and 335 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96-hour growth rate was 206 mg a.i./L.

Visual and Microscopic Observations

After 96 hours of exposure, there were no signs of aggregation (clumping) or adherence of the algae to the test flasks in the negative control or any PFOS treatment group. At 72 and 96 hours of exposure, some cells in the 335 mg a.i./L treatment group appeared small in comparison to the control. All other treatment groups showed no noticeable changes in cell morphology.

Reversibility of Growth Inhibition

The 335 mg a.i./L treatment group was maximally inhibited at the end of the 96-hour exposure period. Aliquots of the test solution were diluted with algal medium and cultured for seven days. Based on the increase in growth observed in the recovery phase, the effect on algal growth was found to be algistatic, rather than aligicidal. Cell densities for the recovery phase are presented in Table 11 and are illustrated graphically in Figure 3.

CONCLUSIONS

The conclusions of this study were based on the most sensitive endpoint measured (i.e., cell density, area under the growth curve and/or growth rate). The 72-hour EC50, based on cell density, was 242 mg a.i./L, with 95% confidence limits of 200 and 276 mg a.i./L. The 96-hour EC50, based on area under the growth curve, was 252 mg a.i./L, with 95% confidence limits of 220 and 285 mg a.i./L. The 72-hour NOAEC, based on cell density and area under the growth curve, was <62.3 mg a.i./L, the lowest concentration tested. The 96-hour NOAEC, based on area under the growth curve, was <62.3 mg a.i./L, the lowest concentration tested. Based on the presence of visible algal growth at recovery termination, PFOS was considered to be algistatic, rather than algicidal, at the concentrations tested.

REFERENCES

1 **U.S. Environmental Protection Agency**. 1996. Series 850 - Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II.*

2 **ASTM Standard Guide 1218-90E.** 1990. *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae*. American Society for Testing and Materials. Philadelphia, Pennsylvania.

3 **The SAS System for Windows**. 1996. Release 6.12, TS Level 0020. SAS Institute Inc., Cary, North Carolina.

4 **West, Inc. and D.D. Gulley**. TOXSTAT Version 3.5. Copyright 1996. Western EcoSystems Technology, Inc., Cheyenne, Wyoming.

Table 1

Summary of Analytical Chemistry Data

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Nominal Concentration (mg a.i./L)	Sampling Time (Hours)	Measured Concentration[1] (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	0[2]	< LOQ	< LOQ	--
	72[3]	< LOQ		
	96[4]	< LOQ		
61.5	0	62.3	62.3	101
	72	63.6		
	96	61.1		
81.3	0	83.8	83.2	102
	72	84.7		
	96	81.0		
110	0	109	111	101
	72	113		
	96	110		
147	0	147	150	102
	72	154		
	96	149		
198	0	209	206	104
	72	209		
	96	199		
264	0	271	266	101
	72	268		
	96	258		
347	0	334	335	96.5
	72	342		
	96	329		
347 (abiotic)	72[5]	341	339	97.7
	96[5]	336		

[1] Limit of Quantitation (LOQ) was 4.39 mg a.i./L.
[2] 0-hour samples were collected from individual batches of test solution prepared for the treatment and control groups for test initiation.
[3] 72-hour samples were collected from the additional analytical replicate.
[4] 96-hour samples were composites of test solution collected from each of the three replicates per treatment and control group.
[5] 72 and 96-hour samples were collected from the additional abiotic replicates.

Table 2

Temperature Measurements

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Time (Day)	Temperature [1] (°C)	
	Measurement 1	Measurement 2
0	23.6	23.9
1	24.5	24.2
2	24.6	24.2
3	24.2	24.1
4	24.0	23.3
5	23.7	23.7
6	23.8	23.4
7	23.3	23.3
8	23.2	23.5
9	23.2	23.1
10	23.5	23.7
11	23.2	23.3

[1] Temperature Measurement 2 was taken at least 4 hours after Measurement 1, with the exception of test initiation and termination (Days 0 and 11).

Table 3

Light Intensity Measurements

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Test Day	Shaker Table	Light Intensity Measurements [1] (lux)				
		No. 1	No. 2	No. 3	No. 4	No.5
0	1	3910	4370	4490	4230	4320
	2	4500	4050	4510	3930	3920

[1] Light intensity was measured at five locations over each of two shaker tables.

Table 4

pH Measurements

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	pH Measurements	
	0 Hours[1]	96 Hours[2]
Negative Control	7.5	8.6
62.3	7.5	8.6
83.2	7.5	8.8
111	7.5	8.9
150	7.5	8.8
206	7.5	8.3
266	7.5	8.2
335	7.5	7.7
339 (abiotic)	--[3]	7.7

[1] 0-hour samples were collected from the batches of test solution prepared for the treatment and control groups at test initiation.

[2] 96-hour samples were collected from the pooled replicates per treatment and control group.

[3] The abiotic replicates were prepared from the test solution used to prepare the 335 mg a.i./L test solution on Day 0.

Table 5

Mean Cell Densities and Percent Inhibition for Each 24-Hour Interval During the Test

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	24 Hours		48 Hours		72 Hours		96 Hours	
	Mean Cell Density[1] (cells/mL)	Percent Inhibition[1]	Mean Cell Density[1] (cells/mL)	Percent Inhibition[1]	Mean Cell Density[1] (cells/mL)	Percent Inhibition[1]	Mean Cell Density[1] (cells/mL)	Percent Inhibition[1]
Negative Control	44,333	--	269,667	--	1,696,667	--	2,726,667	--
62.3	44,333	0	235,000	13	1,126,667*	34	2,366,667	13
83.2	35,333	20	229,333	15	1,190,000*	30	2,366,667	13
111	39,333	11	227,667	16	1,123,333*	34	2,373,333	13
150	37,667	15	248,333	7.9	1,306,667	23	2,473,333	9.3
206	42,333	4.5	217,000	20	1,276,667	25	2,093,333*	23
266	26,333	41	126,667	53	606,667*	64	1,330,000*	51
335	7,000	84	15,333	94	17,333*	99	35,333*	99

[1] Values calculated using SAS 6.12. Manual calculations may differ slightly.
* Indicates a significant difference from the negative control using Dunnett's test ($p \leq 0.05$).

Table 6

Mean Areas Under the Growth Curve and Percent Inhibition for Each 24-Hour Interval During the Test

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Area[1]	Percent Inhibition[1]	Mean Area[1]	Percent Inhibition[1]	Mean Area[1]	Percent Inhibition[1]	Mean Area[1]	Percent Inhibition[1]
Negative Control	412,000	--	3,940,000	--	27,296,000	--	80,136,000	--
62.3	412,000	0.0	3,524,000	11	19,624,000*	28	61,304,000*	24
83.2	304,000	26	3,240,000	18	20,032,000*	27	62,472,000*	22
111	352,000	15	3,316,000	16	19,288,000*	29	61,008,000*	24
150	332,000	19	3,524,000	11	21,944,000	20	67,064,000	16
206	388,000	5.8	3,260,000	17	20,944,000*	23	61,144,000*	24
266	196,000	52	1,792,000	55	10,352,000*	62	33,352,000*	58
335	0	100	32,000	99	184,000*	99	576,000*	99

[1] Values calculated using SAS 6.12. Manual calculations may differ slightly.
* Indicates a significant difference from the negative control using Dunnett's test ($p \leq 0.05$).

Table 7

Mean Growth Rates and Percent Inhibition for Each 24-Hour Interval During the Test

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Growth Rate[1] (cells/mL/hr)	Percent Inhibition[1]	Mean Growth Rate[1] (cells/mL/hr)	Percent Inhibition[1]	Mean Growth Rate[1] (cells/mL/hr)	Percent Inhibition[1]	Mean Growth Rate[1] (cells/mL/hr)	Percent Inhibition[1]
Negative Control	0.0620	--	0.0686	--	0.0711	--	0.0584	--
62.3	0.0618	0.28	0.0657	4.3	0.0653	8.1	0.0569	2.5
83.2	0.0525	15	0.0652	4.9	0.0663	6.7	0.0568	2.7
111	0.0569	8.3	0.0650	5.2	0.0656	7.8	0.0570	2.5
150	0.0544	12	0.0669	2.5	0.0674	5.2	0.0574	1.7
206	0.0600	3.1	0.0640	6.7	0.0672	5.5	0.0556	4.7
266	0.0383	38	0.0518	24	0.0569*	20	0.0506*	13
335	0.0000	100	0.0086	87	0.0068*	90	0.0125*	79

[1] Values calculated using SAS 6.12. Manual calculations may differ slightly.
* Indicates a significant difference from the negative control using Dunnett's test ($p \leq 0.05$).

Table 8

EC Values Based on Cell Density Over the 96-Hour Exposure Period

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Time	EC10 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC50 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC90 (mg a.i./L)	95% Confidence Limits (mg a.i./L)
24 Hours	Not Determined	--	281	214 - 312	Not Determined	--
48 Hours	Not Determined	--	261	219 - 306	Not Determined	--
72 Hours	< 62.3	--[1]	242	200 - 276	317	306 - 326
96 Hours	< 62.3	--[1]	263	217 - 299	322	310 - 328

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

Table 9

EC Values Based on Area Under the Growth Curve Over the 96-Hour Exposure Period

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Time	EC10 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC50 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC90 (mg a.i./L)	95% Confidence Limits (mg a.i./L)
24 Hours	Not Determined	--	262	205 - 308	Not Determined	--
48 Hours	Not Determined	--	259	227 - 303	Not Determined	--
72 Hours	< 62.3	--[1]	246	210 - 277	318	307 - 325
96 Hours	< 62.3	--[1]	252	220 - 285	319	308 - 326

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

Table 10

EC Values Based on Growth Rate Over the 96-Hour Exposure Period

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Time	EC10 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC50 (mg a.i./L)	95% Confidence Limits (mg a.i./L)	EC90 (mg a.i./L)	95% Confidence Limits (mg a.i./L)
24 Hours	Not Determined	--	279	212 - 306	Not Determined	--
48 Hours	Not Determined	--	294	271- 307	Not Determined	--
72 Hours	221	190 - 252	295	288 - 305	335	323 - 335
96 Hours	243	209 - 295	305	295 - 316	> 335	--[1]

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

Table 11

Cell Densities During the Recovery Phase

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	Cell Densities (cells/mL)			
	Day 0[2]	Day 3	Day 6	Day 7
Negative Control	15,000	2,280,000	2,560,000	2,520,000
335[1]	5,000	7,000	179,000	780,000

[1] The treatment group was diluted to a concentration of the test substance that theoretically would not inhibit growth.

[2] Due to the method defined in the protocol used to prepare recovery phase test solutions, initial cell densities were not equivalent in both groups.

Figure 1. Negative control algal growth, expressed as cell density, during the 96-hour exposure period.

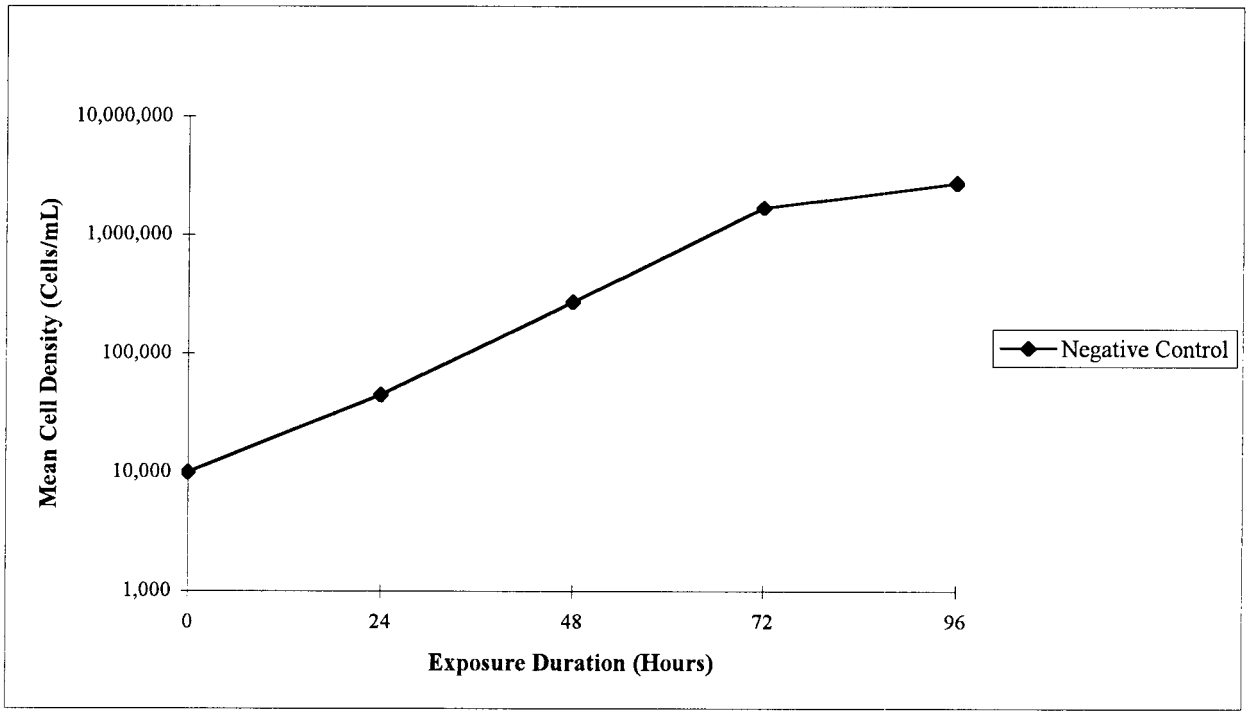


Figure 2. Concentration-response curve, expressed as cell density, over the 96-hour exposure period.

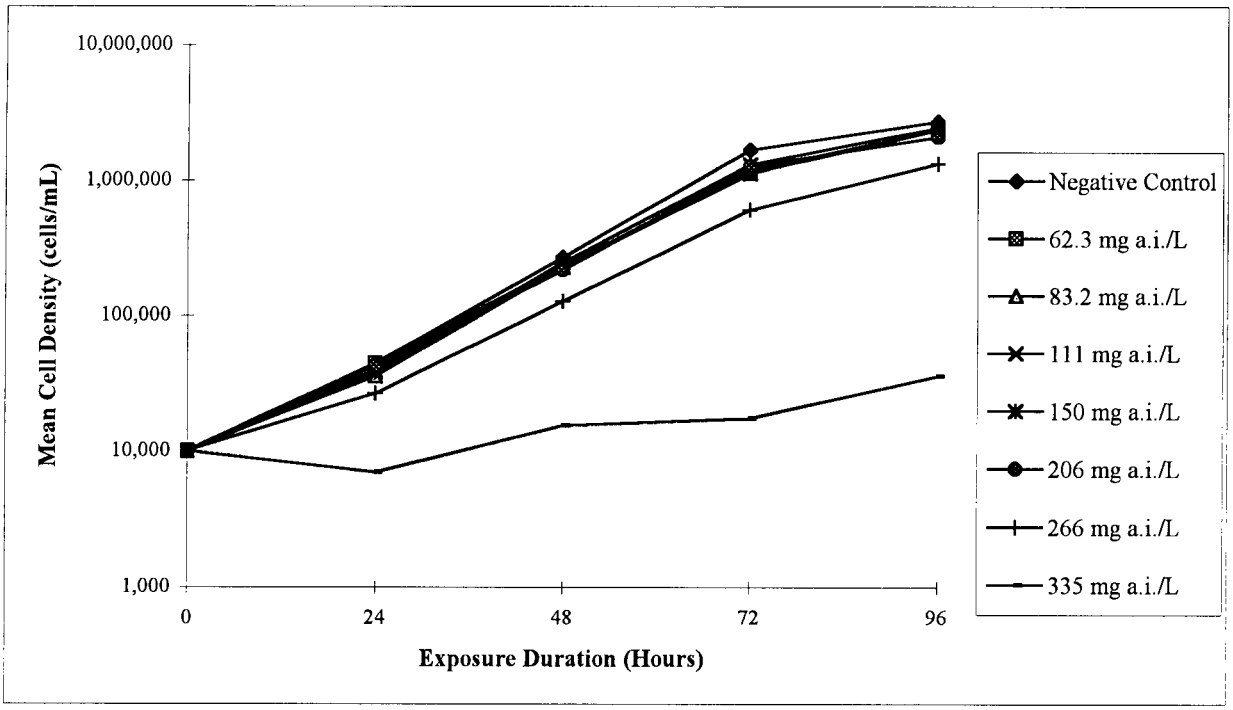
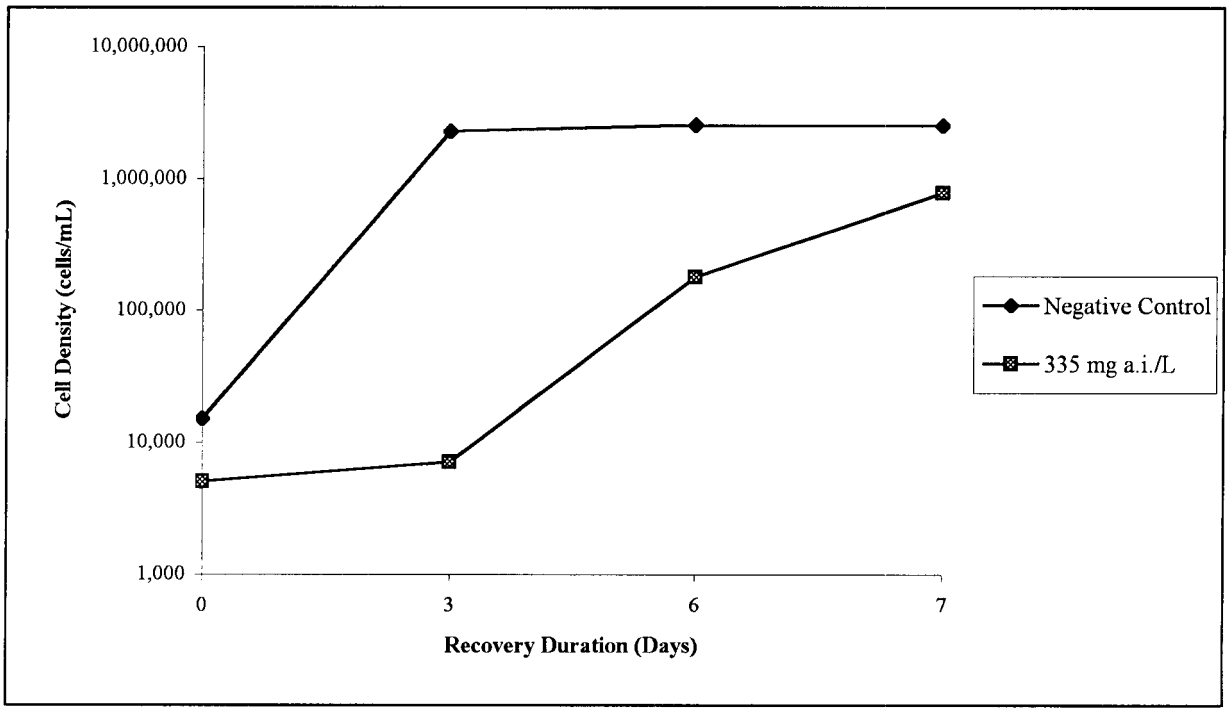


Figure 3. Recovery-phase response curve, expressed as cell density.



APPENDIX I

Freshwater Algal Medium with Silica Constituents[1]

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Diatom, *Navicula pelliculosa*
Dilution Water:	Freshwater Algal Medium with Silica and Selenium

Compound	Nominal Concentration	
$MgCl_2 \cdot 6H_2O$	12.16	mg/L
$CaCl_2 \cdot 2H_2O$	4.40	mg/L
H_3BO_3	0.1856	mg/L
$MnCl_2 \cdot 4H_2O$	0.416	mg/L
$ZnCl_2$	3.28	µg/L
$FeCl_3 \cdot 6H_2O$	0.1598	mg/L
$CoCl_2 \cdot 6H_2O$	1.428	µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26	µg/L
$CuCl_2 \cdot 2H_2O$	0.012	µg/L
$Na_2EDTA \cdot 2H_2O$	0.300	mg/L
$NaNO_3$	25.50	mg/L
$MgSO_4 \cdot 7H_2O$	14.70	mg/L
K_2HPO_4	1.044	mg/L
$NaHCO_3$	15.0	mg/L
$Na_2SiO_3 \cdot 9H_2O$	20.0	mg/L
$Na_2SeO_3 \cdot 5H_2O$	0.010	mg/L

[1] The pH was adjusted to 7.5 ± 0.1 using 10% HCl and 0.1 N NaOH.

APPENDIX II

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

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

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

APPENDIX III

THE ANALYSIS OF PFOS IN FRESHWATER ALGAL MEDIUM

IN SUPPORT OF

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-112

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Diatom (*Navicula pelliculosa*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-112

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

3/26/01
DATE

MANAGEMENT:

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

3/26/01
DATE

Introduction

Freshwater algal medium samples were collected from a 96-hour toxicity test designed to determine the effects of PFOS (Perfluorooctanesulfonate, Potassium Salt) to the freshwater diatom (*Navicula pelliculosa*). This study was conducted by Wildlife International, Ltd. and identified as Project No.: 454A-112. 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 February 25 and 29, 2000 and were 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 algal medium 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 centrifuged, as necessary, and diluted in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (50 mm × 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 0.0439 to 0.879 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 4.39 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.0439 mg a.i./L) and the dilution factor of the matrix blank samples (100).

Matrix Blank and Fortification Samples

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

Freshwater algal medium was fortified at 17.6, 176 and 351 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 108%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-112-5, nominal concentration of 147 mg a.i./L in freshwater algal medium.

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: 0.100 mL

Final Volume: 50.0 mL

Dilution Factor: 500

PFOS Peak Area: 8477265

Internal Standard Peak Area: 1849815

Peak Area Ratio: 4.58276

Calibration curve equation.

Slope: 1.44418

Intercept: 0.32839

Curve is weighted (1/x)

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

$$= \frac{4.58276 - 0.32839}{1.44418} \times 0.100$$

$$= 0.2946$$

PFOS (mg a.i./L) in sample = PFOS (mg a.i./L) at instrument × Dilution Factor

$= 0.2946 \times 500$

$= 147$

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

$$= \frac{147}{147} \times 100$$

$= 100\%$

Quantitation software for recoveries: MacQuan, version 1.6.

RESULTS

Sample Analysis

Freshwater algal medium samples were collected from the 96-hour toxicity test with the freshwater diatom (*Navicula pelliculosa*) at test initiation, February 25, 2000 (Day 0), on February 28, 2000 (Day 3) and at test termination, February 29, 2000 (Day 4). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 96.2 to 106% of the nominal concentrations. Samples collected at Day 3 had a measured concentration range of 98.2 to 106% of nominal values. Samples collected at test termination (Day 4) had a measured concentration range of 94.8 to 101% of nominal values (Table 4). Samples from the abiotic 347 mg a.i./L treatment group were comparable to samples from the 347 mg a.i./L treatment group with the freshwater diatom present (Table 4). A representative ion chromatogram of a test sample is shown in Figure 7.

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 × 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:	5.0 μL
PFOS RETENTION TIME:	Approximately 3.7 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 2.6 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of PFOS[1] (mg a.i./L)
Number (454A-112-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ

[1] The limit of quantitation (LOQ) was 4.39 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0439 mg a.i./L) and the dilution factor of the matrix blank samples (100).

Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-112-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered[2]
	Fortified[1]	Measured[1]	
MAS-1	17.6	20.9	119
MAS-4	17.6	20.3	115
MAS-7	17.6	20.4	116
MAS-2	176	192	109
MAS-5	176	187	106
MAS-8	176	185	105
MAS-3	351	359	102
MAS-6	351	349	99.2
MAS-9	351	352	100
		Mean	= 108
		Standard Deviation	= 7.29
		CV	= 6.76%
		N	= 9

[1] Concentrations were corrected for change in test substance purity (98.9% to 86.9%) per Certificate of Analysis dated September 7, 2000.

[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since fortified and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

Table 4

Measured Concentrations of PFOS in Freshwater Algal Medium Samples from a Freshwater Diatom (*Navicula pelliculosa*) 96-Hour Toxicity Test

Nominal Test Concentration[1] (mg a.i./L)	Sample Number (454A-112-)	Sampling Time (Day)	PFOS Measured Concentration[1,2] (mg a.i./L)	Percent of Nominal[3]
0.0	1	0	< LOQ	--
(Negative Control)	9	3	< LOQ	--
	18	4	< LOQ	--
61.5	2	0	62.3	101
	10	3	63.6	103
	19	4	61.1	99.3
81.3	3	0	83.8	103
	11	3	84.7	104
	20	4	81.0	99.7
110	4	0	109	99.6
	12	3	113	103
	21	4	110	99.7
147	5	0	147	100
	13	3	154	105
	22	4	149	101
198	6	0	209	106
	14	3	209	106
	23	4	199	101
264	7	0	271	103
	15	3	268	102
	24	4	258	97.7
347	8	0	334	96.2
	16	3	342	98.5
	25	4	329	94.8
347	17	3	341	98.2
(Abiotic)	26	4	336	96.8

[1] Concentrations were corrected for change in test substance purity (98.9% to 86.9%) per Certificate of Analysis dated September 7, 2000.

[2] The limit of quantitation (LOQ) was 4.39 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0439 mg a.i./L) and the dilution factor of the matrix blank samples (100).

[3] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly since nominal and measured concentrations were corrected for change in test substance purity and rounded for reporting purposes.

METHOD OUTLINE FOR THE ANALYSIS OF PFOS IN FRESHWATER ALGAL MEDIUM

Prepare matrix fortification samples by spiking the requisite volume of PFOS stock solutions directly into freshwater algal medium using gas-tight syringes and Class A volumetric flasks.

↓

Centrifuge all samples, as necessary, for approximately five minutes at approximately 1500 rpm.

↓

Dilute matrix fortification and test samples into the range of the calibration standards by partially filling Class A volumetric flasks with 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v). Add the appropriate volume of sample and bring the flask to volume with the dilution solvent. Process the matrix blank sample using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.

↓

Ampulate samples and submit for LCMS analysis.

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

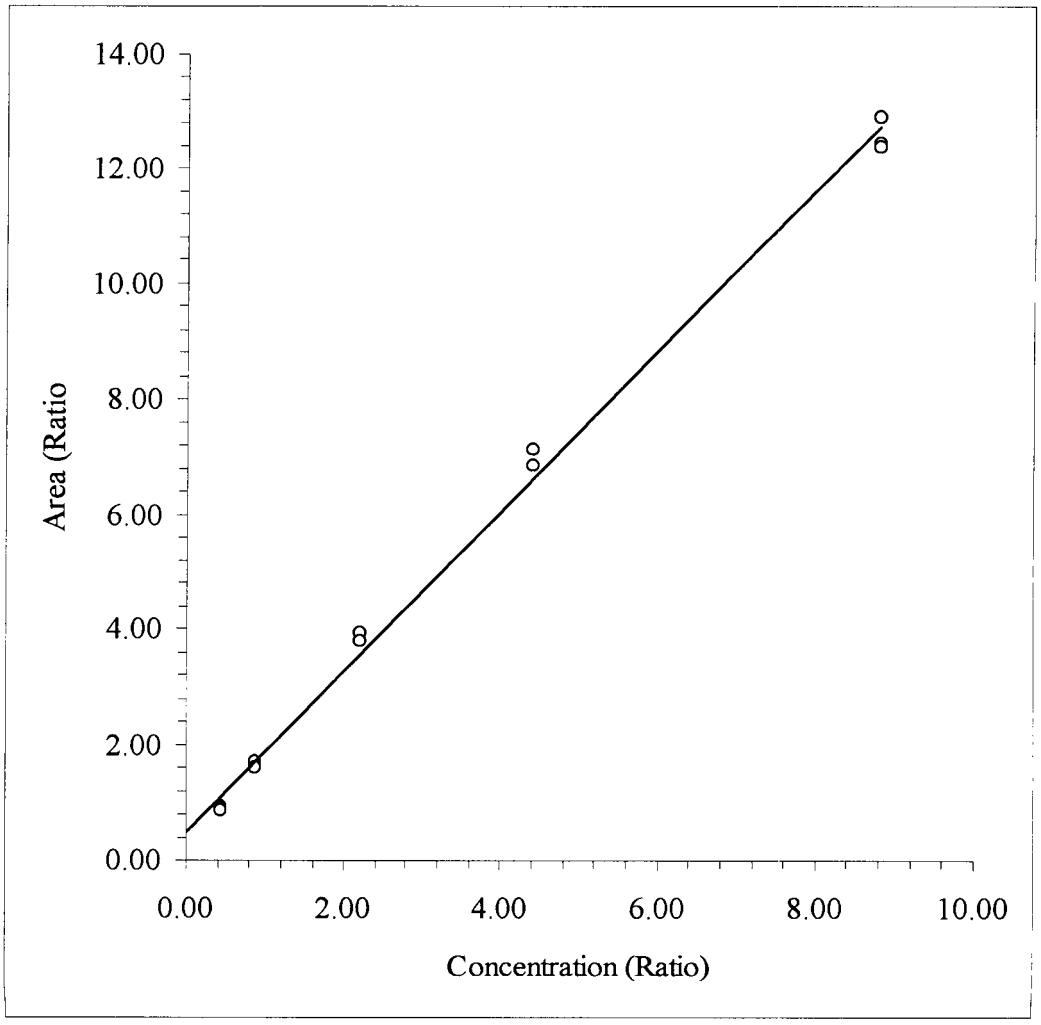


Figure 2. A typical calibration curve for PFOS. Slope = 1.44418; Intercept = 0.32839; r = 0.9975. Curve is weighted (1/x).

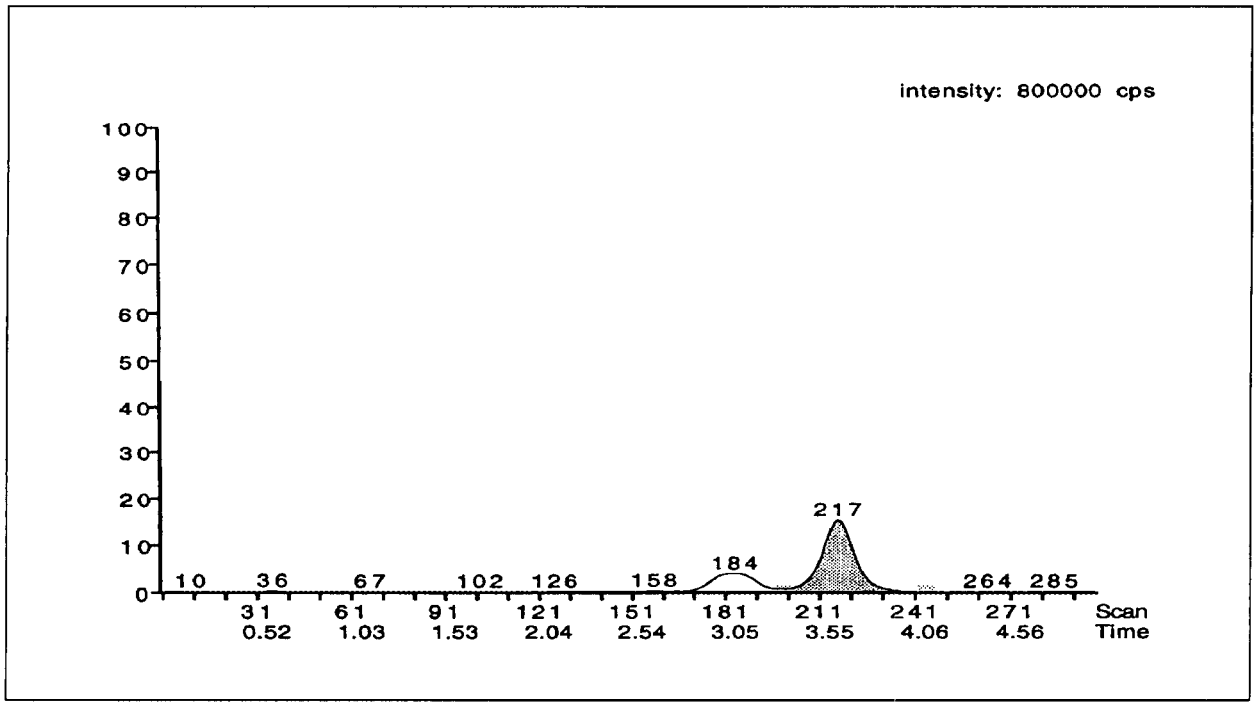


Figure 3. A representative ion chromatogram of a low-level (0.0439 mg a.i./L) PFOS standard.

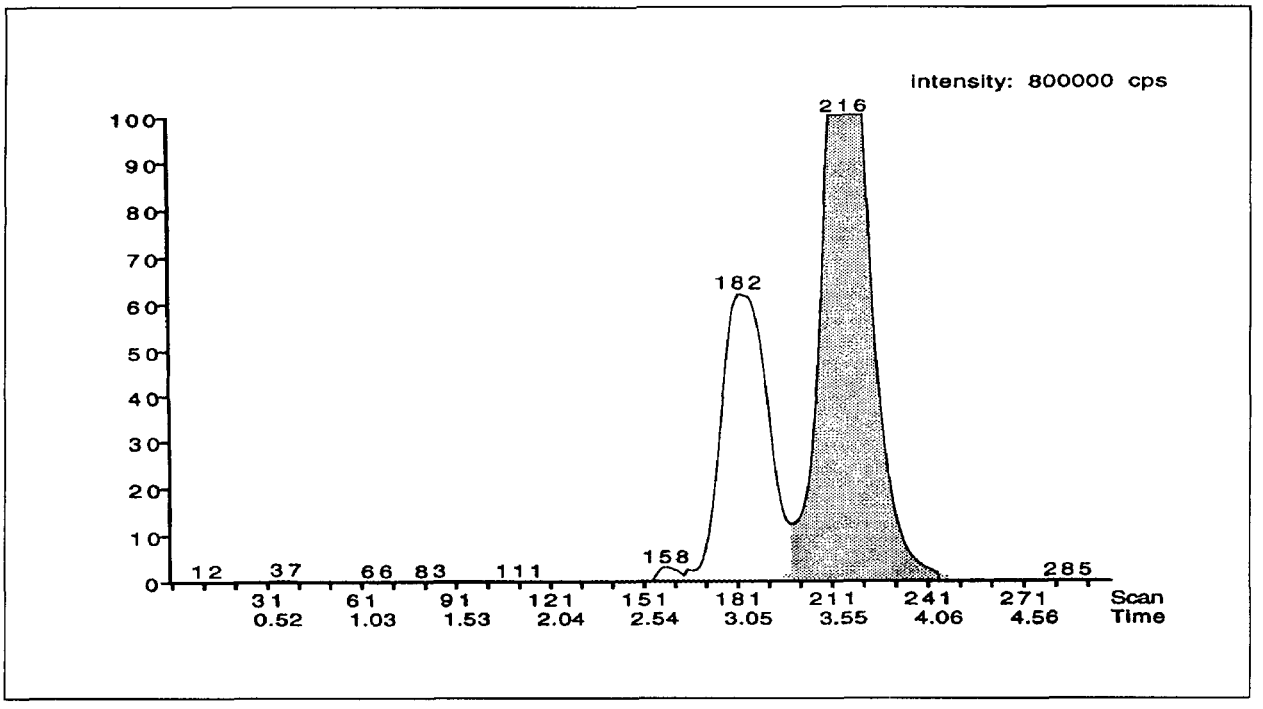


Figure 4. A representative ion chromatogram of a high-level (0.879 mg a.i./L) PFOS standard.

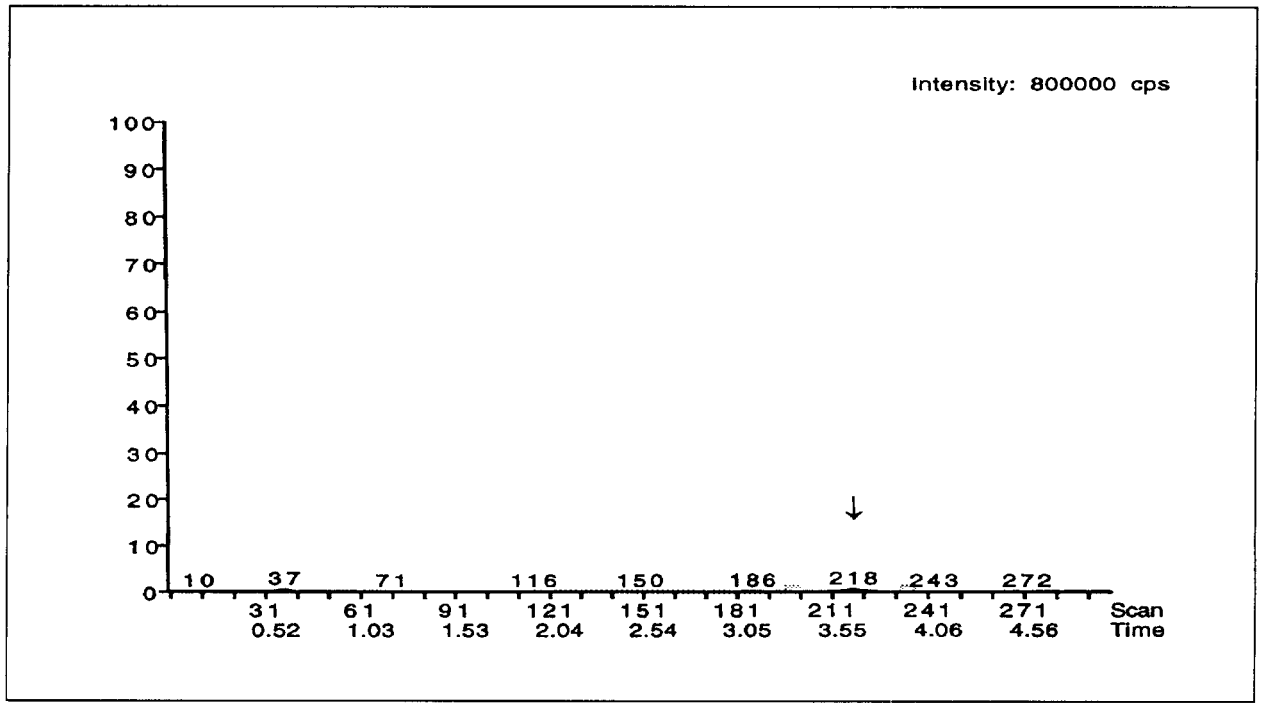


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-112-MAB-1). The arrow indicates the retention time of PFOS.

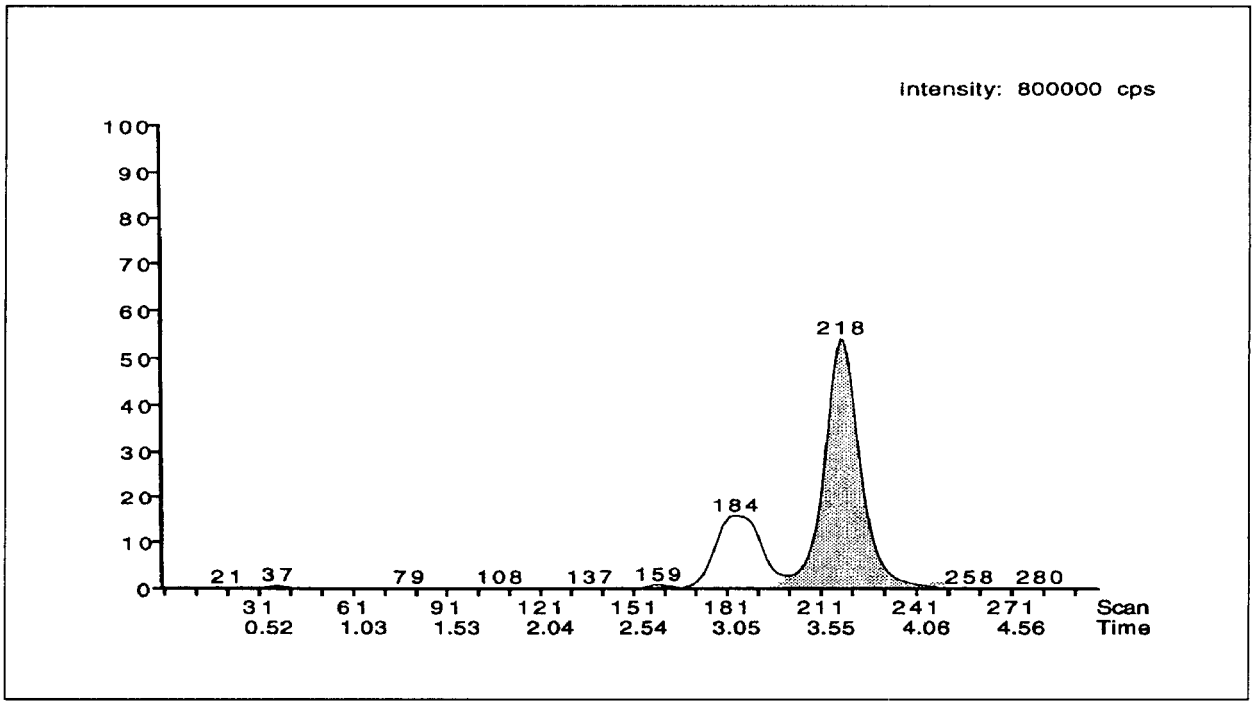


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-112-MAS-1).

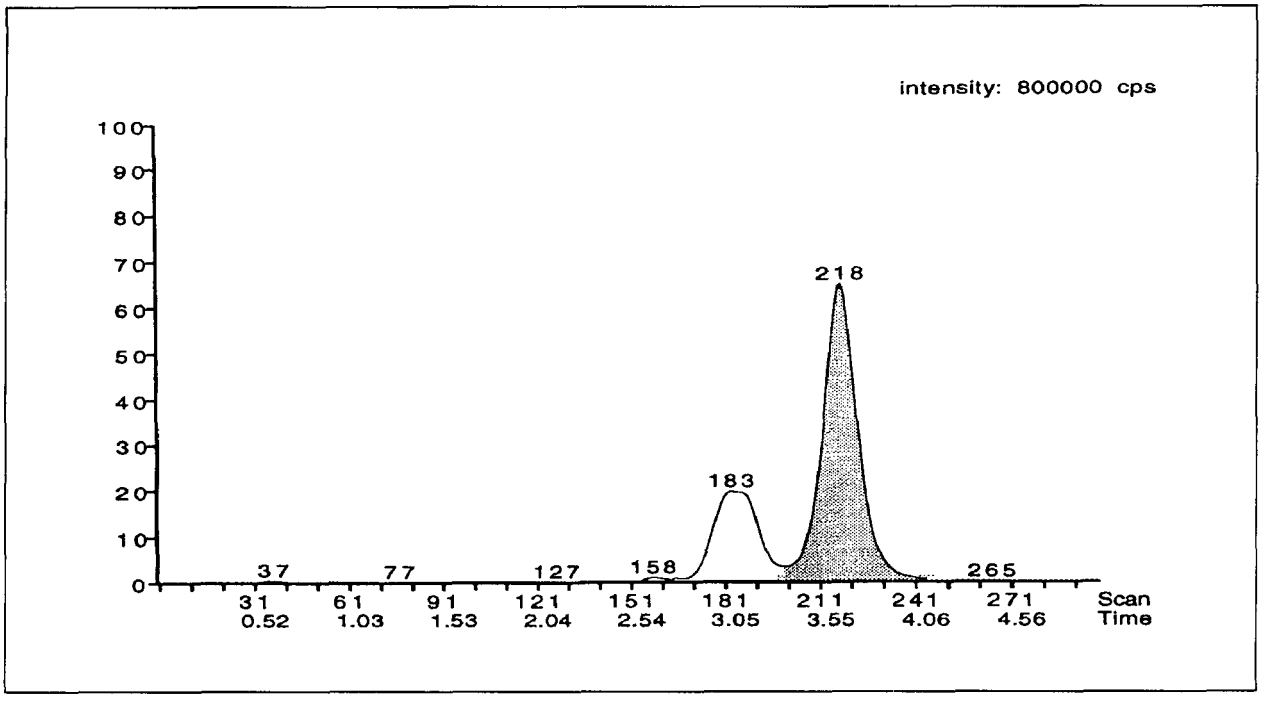


Figure 7. A representative ion chromatogram of a test sample (454A-112-2).

APPENDIX IV

Cell Density for Each Replicate Per Treatment Over the 96-Hour Exposure Period

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	Replicate	Cell Densities (Cells/mL)[1]			
		24 Hours	48 Hours	72 Hours	96 Hours
Negative Control	A	47,000	276,000	1,370,000	2,980,000
	B	45,000	249,000	1,620,000	2,500,000
	C	41,000	284,000	2,100,000	2,700,000
62.3	A	46,000	271,000	1,120,000	2,200,000
	B	38,000	218,000	1,410,000	2,600,000
	C	49,000	216,000	850,000	2,300,000
83.2	A	33,000	220,000	1,320,000	2,580,000
	B	39,000	251,000	1,190,000	2,640,000
	C	34,000	217,000	1,060,000	1,880,000
111	A	42,000	216,000	1,090,000	2,500,000
	B	34,000	212,000	1,070,000	2,440,000
	C	42,000	255,000	1,210,000	2,180,000
150	A	39,000	259,000	1,630,000	2,480,000
	B	28,000	240,000	1,020,000	2,460,000
	C	46,000	246,000	1,270,000	2,480,000
206	A	46,000	246,000	1,550,000	2,140,000
	B	42,000	207,000	1,130,000	1,900,000
	C	39,000	198,000	1,150,000	2,240,000
266	A	34,000	172,000	670,000	1,710,000
	B	29,000	78,000	520,000	890,000
	C	16,000	130,000	630,000	1,390,000
335	A	9,000	18,000	14,000	53,000
	B	7,000	12,000	26,000	31,000
	C	5,000	16,000	12,000	22,000

[1] The initial cell density of the stock culture was determined and an inoculum volume was administered to each test chamber to yield a cell density of approximately 10,000 cells/mL at test initiation (0 hours).

APPENDIX V

Area Under the Growth Curve for Each Replicate Per Treatment Over the 96-Hour Exposure Period

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	Replicate	Cumulative Area Under the Growth Curve			
		0 – 24 Hours	0 - 48 Hours	0 - 72 Hours	0 - 96 Hours
Negative Control	A	444,000	4,080,000	23,592,000	75,552,000
	B	420,000	3,708,000	25,896,000	75,096,000
	C	372,000	4,032,000	32,400,000	89,760,000
62.3	A	432,000	3,996,000	20,448,000	60,048,000
	B	336,000	3,168,000	22,464,000	70,344,000
	C	468,000	3,408,000	15,960,000	53,520,000
83.2	A	276,000	3,072,000	21,312,000	67,872,000
	B	348,000	3,588,000	20,640,000	66,360,000
	C	288,000	3,060,000	18,144,000	53,184,000
111	A	384,000	3,240,000	18,672,000	61,512,000
	B	288,000	3,000,000	18,144,000	60,024,000
	C	384,000	3,708,000	21,048,000	61,488,000
150	A	348,000	3,684,000	26,112,000	75,192,000
	B	216,000	3,192,000	18,072,000	59,592,000
	C	432,000	3,696,000	21,648,000	66,408,000
206	A	432,000	3,696,000	25,008,000	69,048,000
	B	384,000	3,132,000	18,936,000	55,056,000
	C	348,000	2,952,000	18,888,000	59,328,000
266	A	288,000	2,520,000	12,384,000	40,704,000
	B	228,000	1,272,000	8,208,000	24,888,000
	C	72,000	1,584,000	10,464,000	34,464,000
335	A	0	84,000	228,000	792,000
	B	0	0	216,000	660,000
	C	0	12,000	108,000	276,000

APPENDIX VI

Growth Rate for Each Replicate Per Treatment Over the 96-Hour Exposure Period

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Freshwater Diatom, *Navicula pelliculosa*
Dilution Water: Freshwater Algal Medium with Silica and Selenium

Mean Measured Concentration (mg a.i./L)	Replicate	Growth Rate			
		0 – 24 Hours	0 – 48 Hours	0 - 72 Hours	0 - 96 Hours
Negative Control	A	0.0645	0.0691	0.0683	0.0593
	B	0.0627	0.0670	0.0707	0.0575
	C	0.0588	0.0697	0.0743	0.0583
62.3	A	0.0636	0.0687	0.0655	0.0562
	B	0.0556	0.0642	0.0687	0.0579
	C	0.0662	0.0640	0.0617	0.0566
83.2	A	0.0497	0.0644	0.0678	0.0578
	B	0.0567	0.0671	0.0664	0.0581
	C	0.0510	0.0641	0.0648	0.0545
111	A	0.0598	0.0640	0.0652	0.0575
	B	0.0510	0.0636	0.0649	0.0573
	C	0.0598	0.0675	0.0666	0.0561
150	A	0.0567	0.0678	0.0707	0.0574
	B	0.0429	0.0662	0.0642	0.0573
	C	0.0636	0.0667	0.0673	0.0574
206	A	0.0636	0.0667	0.0700	0.0559
	B	0.0598	0.0631	0.0657	0.0547
	C	0.0567	0.0622	0.0659	0.0564
266	A	0.0510	0.0593	0.0584	0.0536
	B	0.0444	0.0428	0.0549	0.0468
	C	0.0196	0.0534	0.0575	0.0514
335	A	0.0000	0.0122	0.0047	0.0174
	B	0.0000	0.0038	0.0133	0.0118
	C	0.0000	0.0098	0.0025	0.0082

APPENDIX VII

Changes to Protocol

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

1. The protocol was amended to add the proposed experimental start and termination dates, test concentrations and test substance identification number.

2. The protocol was amended to clarify that the nominal concentration may exceed 100 mg/L.

3. The protocol was amended to add a recovery phase to the study.

4. The nominal test concentrations were a series of seven concentrations, and were recalculated based on a test substance purity of 90.49%.

5. The nominal test concentrations were a series of seven concentrations, and were recalculated based on a test substance purity of 86.9%.

6. The test solutions were prepared individually, rather than by dilution of a primary or secondary stock solution.

7. Two abiotic replicates, rather than one, were prepared at the highest concentration, with one replicate sampled on each of Days 3 and 4.

8. The protocol was amended to correct typographical errors in the units of measurement for some of the components of the algal medium.

9. The protocol was amended to change the test substance name from Perfluorooctane Sulfonic Acid, Potassium Salt to Perfluorooctanesulfonate, Potassium Salt.

APPENDIX VIII

Personnel Involved in the Study

The following key 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., Director, Analytical Chemistry
3. Raymond L. Van Hoven, Ph.D., Scientist
4. Cary A. Sutherland, Laboratory Supervisor
5. Debbie Desjardins, Biologist