

AR226-0085

PFOS:
A 96-HOUR TOXICITY TEST WITH THE
FRESHWATER ALGA (*Selenastrum capricornutum*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-103A

3M LAB REQUEST NO. U2723

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.5400
and
OECD Guideline 201

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STUDY INITIATION DATE: December 4, 1998

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AMENDED REPORT DATE: April 26, 2000

Submitted to

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-103A

STUDY COMPLETION: June 4, 1999

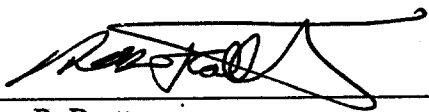
REPORT AMENDED: April 26, 2000

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, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992 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.

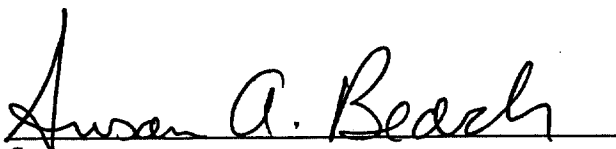
The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:


Kurt R. Drott
Senior Biologist

4/26/00
DATE

SPONSOR:


Susan A. Beach
Sponsor

4/27/00
DATE

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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, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; 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:
<u>Initial Trial 454A-103</u>			
Test Substance Preparation	February 18, 1999	February 18, 1999	February 19, 1999
Instrument Set-up	February 22 and 23, 1999	February 23, 1999	February 24, 1999
Draft Report and Data	March 29, 1999	March 29, 1999	March 30, 1999
<u>Definitive Trial 454A-103A</u>			
Test Substance Preparation	April 9, 1999	April 9, 1999	April 12, 1999
Draft Report and Data	May 10 and 11, 1999	May 11, 1999	May 12, 1999
Analytical Data and Draft Report	May 10 and 11, 1999	May 11, 1999	May 11, 1999
Final Report	June 4, 1999	June 4, 1999	June 4, 1999
Amended Report	April 19 and 20, 2000	April 20, 2000	April 24, 2000



Timothy A. Springer, Ph.D.
Manager, Regulatory and Technical Support

4/24/00
DATE

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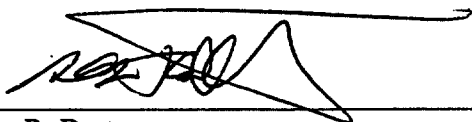
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-103A

STUDY DIRECTOR:



Kurt R. Drott
Senior Biologist

4/26/00

DATE

MANAGEMENT:



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Director, Aquatic Toxicology
and Non-Target Plants

4/26/00

DATE

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SUMMARY

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-103A
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: A 96-Hour Toxicity Test with the Freshwater Alga (<i>Selenastrum capricornutum</i>)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 5.5, 11, 21, 44, 86 and 179 mg a.i./L
TEST DATES:	Experimental Start - April 12, 1999 Exposure Termination - April 16, 1999 Experimental Termination - April 21, 1999
LENGTH OF EXPOSURE:	96 Hours

TEST ORGANISM:	Freshwater Alga (<i>Selenastrum capricornutum</i>)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. Easton, Maryland 21601

CELL DENSITY	
72-HOUR EC50:	70 mg a.i./L
95% CONFIDENCE LIMITS:	44 and 78 mg a.i./L
96-HOUR EC10:	49 mg a.i./L
95% CONFIDENCE LIMITS:	43 and 50 mg a.i./L
96-HOUR EC50:	71 mg a.i./L
95% CONFIDENCE LIMITS:	66 and 73 mg a.i./L
96-HOUR EC90:	137 mg a.i./L
95% CONFIDENCE LIMITS:	105 and 153 mg a.i./L
72-HOUR NOAEC:	44 mg a.i./L
96-HOUR NOAEC:	44 mg a.i./L

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SUMMARY (Continued)

AREA UNDER THE GROWTH
CURVE

72-HOUR EC50:	74 mg a.i./L
95% CONFIDENCE LIMITS:	55 and 82 mg a.i./L
96-HOUR EC10:	49 mg a.i./L
95% CONFIDENCE LIMITS:	40 and 50 mg a.i./L
96-HOUR EC50:	71 mg a.i./L
95% CONFIDENCE LIMITS:	67 and 74 mg a.i./L
96-HOUR EC90:	145 mg a.i./L
95% CONFIDENCE LIMITS:	125 and 155 mg a.i./L
72-HOUR NOAEC:	44 mg a.i./L
96-HOUR NOAEC:	44 mg a.i./L

GROWTH RATE

72-HOUR EC50:	120 mg a.i./L
95% CONFIDENCE LIMITS:	103 and 132 mg a.i./L
96-HOUR EC10:	59 mg a.i./L
95% CONFIDENCE LIMITS:	54 and 63 mg a.i./L
96-HOUR EC50:	126 mg a.i./L
95% CONFIDENCE LIMITS:	115 and 138 mg a.i./L
96-HOUR EC90:	>179 mg a.i./L
95% CONFIDENCE LIMITS:	Not Calculable
72-HOUR NOAEC:	44 mg a.i./L
96-HOUR NOAEC:	44 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from April 12, 1999 to April 21, 1999. Raw data generated by Wildlife International Ltd and a copy of the final report are filed under Project Number 454A-103A in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the toxicity of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the growth of the freshwater alga, *Selenastrum capricornutum*, during a 96-hour exposure period.

EXPERIMENTAL DESIGN

The freshwater alga, *Selenastrum capricornutum*, was exposed to a geometric series of six 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 was also maintained for analytical sampling on Day 3 of the test. In addition, one "abiotic" replicate (test solution without algae) was 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 and definitive tests. The nominal test concentrations selected were 5.7, 11, 23, 46, 91 and 183 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

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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 according to the procedures outlined in the protocol, "PFOS: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)". 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) and OECD Guidelines for Testing of Chemicals, 201 *Algal, Growth Inhibition Test* (2).

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 March 9, 2000 indicated a purity of 90.49%. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 5.7, 11, 23, 46, 91 and 183 mg a.i./L, based on a test substance purity of 90.49%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A primary stock solution was prepared in algal

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medium at a concentration of 183 mg a.i./L. The primary stock solution was stirred with a magnetic stir plate for approximately 24 hours to aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with algal medium to prepare the five additional test concentrations. All final test solutions appeared clear and colorless.

Test Organism

The freshwater alga, *Selenastrum capricornutum*, 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). OECD guidelines recommend that control cell growth should have increased by a factor of at least 16 within 3 days.

Culture Medium

The algal cells were cultured and tested in freshwater algal medium (3). 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 the appropriate volumes of stock nutrient solutions to purified well water (Appendix I). The pH of the medium was adjusted to 7.5 ± 0.1 using 10% HCl and the medium was sterilized by filtration ($0.22 \mu\text{m}$) prior to use. Analyses were performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the well water. The results of analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix II.

Test Apparatus

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

Environmental Conditions

Test flasks were held in an environmental chamber at a temperature of $24 \pm 2^\circ\text{C}$. 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 ± 430 lux. Light intensity was measured at the four corners and the middle of the shaker table daily. Light intensity was measured using an 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 counted immediately. 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.

All 96 hour cell count samples were examined microscopically for atypical cell morphology (e.g., changes in cell shape, size or color). Growth of cells in the replicate test chambers also was assessed for aggregations or flocculations of cells and adherence of the cells to the test chamber.

Statistical Analyses

Cell densities, area under the growth curve values, growth rates and percent inhibition values were calculated using "The SAS System for Windows", Release 6.12 (4). Area under the growth curve was calculated for the control and treatment group using the following formula:

$$A = ((N_1 - N_0)/2)(t_1) + ((N_1 + N_2 - 2N_0)/2)(t_2 - t_1) + ((N_{n-1} + N_n - 2N_0)/2)(t_n - t_{n-1})$$

where: A = Area

N_0 = Nominal number of cells/mL at t_0

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:

$$\text{Growth Rate} = \frac{\ln (N_n/N_0)}{t_n}$$

where: N_0 = Mean measured number of cells/mL at t_0

N_n = Mean measured number of cells/mL at t_n

t_n = Time of n^{th} measurement after beginning of test (hours)

Percent inhibition values were 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:

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$$\text{Percent Inhibition} = \frac{\text{Mean Cell Density}_{\text{Control}} - \text{Mean Cell Density}_{\text{Treatment}}}{\text{Mean Cell Density}_{\text{Control}}} \times 100$$

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 using TOXSTAT Version 3.5 (5). 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 Bartlett's test, respectively. The treatment groups were then compared to the control using Dunnett's test. Results of the statistical analyses 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 replicates for each treatment and the control group. The samples were placed in plastic (Nalgene®) bottles and were analyzed immediately without storage. 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 selected for use in this study were 5.7, 11, 23, 46, 91 and 183 mg a.i./L. Samples collected at the beginning of the test had measured concentrations that ranged from 82 to 98% of nominal. Samples collected at 72 hours and test termination had recoveries that ranged from

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82 to 105% and 90 to 103% of nominal, respectively. When the values obtained for test initiation, at 72 hours and at test termination were averaged, the mean measured test concentrations were 5.5, 11, 21, 44, 86 and 179 mg a.i./L.

Observations and Measurements

Measurements of temperature and light intensity are presented in Tables 2 and 3, respectively. The temperatures ranged from 23.6 to 25.8°C and were within the range established for the test ($24 \pm 2^\circ\text{C}$). The light intensity ranged from 3870 to 4540 lux and was within the desired range for the test (approximately 3870 to 4730 lux) (Table 3). Measurements of pH were 7.4 to 7.5 on Day 0 and ranged from 7.5 to 8.4 at 96 hours (Table 4). The pH of the "abiotic" replicate at test termination was 7.4.

The effect of PFOS upon *Selenastrum capricornutum* was determined by evaluating differences in cell densities, areas under the growth curve and growth rates. Mean values were used to calculate growth inhibition for each 24-hour period. Mean cell densities, areas under the growth curve and growth rates and their corresponding percent inhibition values are presented in Tables 5, 6 and 7, respectively. Cell density, area under the growth curve and growth rate values for each individual replicate are presented in Appendices IV, V and VI, respectively. EC10, EC50 and EC90 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). The coefficient of variation for control cell density was 4%. After 72 hours of exposure, cell density percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -5.2, -1.9, -5.5, 11, 72 and 97%, respectively. Dunnett's test showed that cell density was significantly reduced in the 86 and 179 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 44 mg a.i./L. After 96 hours of exposure, cell density percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -11, -5.1, -18, -12, 77 and 99%, respectively. Dunnett's test showed that cell density was significantly reduced in the 86 and 179 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96 hour cell density was 44 mg a.i./L.

After 72 hours of exposure, area under the growth curve percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -2.9, 1.3, -3.8, 5.8, 68 and 94%, respectively. Dunnett's test showed that area under the growth curve was significantly reduced in the 86 and 179 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 72 hour area under the growth curve was 44 mg a.i./L. After 96 hours of exposure, area under the growth curve percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -8.5, -3.3, -13, -5.3, 75 and 98%, respectively. Dunnett's test showed that area under the growth curve was significantly reduced in the 86 and 179 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96 hour area under the growth curve was 44 mg a.i./L.

After 72 hours of exposure, growth rate percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -1.1, -0.68, -0.72, 3.4, 31 and 82%, respectively. Dunnett's test showed that growth rate was significantly reduced in the 86 and 179 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 72 hour growth rate was 44 mg a.i./L. After 96 hours of exposure, growth rate percent inhibition in the 5.5, 11, 21, 44, 86 and 179 mg a.i./L treatment groups was -1.9, -0.84, -3.0, -2.0, 27 and 79%, respectively. Dunnett's test showed that growth rate was significantly reduced in the 86 and 179 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96 hour growth rate was 44 mg a.i./L.

Visual and Microscopic Observations

After 96 hours of exposure, there were no signs of aggregation, flocculation or adherence of the algae to the test flasks in the negative control or any PFOS treatment group. In addition, there were no noticeable changes in cell color or morphology when compared to the negative control, although a few cells appeared enlarged in the 86 and 179 mg a.i./L treatment group.

Reversibility of Growth Inhibition

The 179 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 five days. Based on the growth observed in the recovery phase, the effect on algal growth was found to be algistatic. 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 70 mg a.i./L with 95% confidence limits of 44 and 78 mg a.i./L. The 96-hour EC10, based on cell density, was 49 mg a.i./L with 95% confidence limits of 43 and 50 mg a.i./L. The 96-hour EC50, based on cell density, was 71 mg a.i./L with 95% confidence limits of 66 and 73 mg a.i./L. The 96-hour EC90, based on cell density, was 137 mg a.i./L with 95% confidence limits of 105 and 153 mg a.i./L. The 72-hour and 96-hour NOAEC values, based on cell density, area under the growth curve and growth rate, were 44 mg a.i./L. 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 Organization of Economic Cooperation and Development. 1984. *Algal, Growth Inhibition Test*. OECD Guideline for Testing of Chemicals. Guideline 201. Paris.
- 3 ASTM Standard Guide 1218-90E, *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae*. August 1990.
- 4 The SAS System for Windows. 1996. Release 6.12, TS Level 0020. SAS Institute Inc., Cary, North Carolina.
- 5 West, Inc. and D.D. Gulley. TOXSTAT Version 3.5. Copyright 1996. Western EcoSystems Technology, Inc., Cheyenne, Wyoming.

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Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>			
Dilution Water:	Freshwater Algal Medium			
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	--	--
	72 ³	< LOQ		
	96 ⁴	< LOQ		
5.7	0	4.73	5.5	96
	72	6.04		
	96	5.84		
11	0	10.7	11	100
	72	11.2		
	96	12.1		
23	0	19.8	21	91
	72	23.1		
	96	20.7		
46	0	42.7	44	96
	72	41.9		
	96	46.3		
91	0	83.3	86	95
	72	86.0		
	96	88.3		
183	0	179	179	98
	72	186		
	96	172		
183	72 ⁵	150	169	92
	96 ⁵	188		

¹Limit of Quantitation (LOQ) was 0.115 mg a.i./L.²0-hour samples were collected from individual batches of test solution prepared for the treatment and control groups at test initiation.³72-hour samples were collected from the additional (D) replicate (biotic).⁴96-hour samples were composites of test solution collected from each of the three replicates per treatment and control group.⁵72 and 96 hour sample collected from the additional (E) replicate (abiotic).

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Table 2

Temperature Measurements

Sponsor:	3M Corporation	
Test Substance:	PFOS	
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>	
Dilution Water:	Freshwater Algal Medium	
Time (Day)	Temperature (°C)	
	Measurement 1	Measurement 2
0	23.6	24.2
1	24.8	24.2
2	24.8	24.0
3	25.1	25.3
4	25.8	24.9
5	25.7	24.4
6	25.6	24.9
7	24.0	24.0
8	23.9	23.8
9	23.9	23.9

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Table 3

Light Intensity Measurements

Sponsor:	3M Corporation				
Test Substance:	PFOS				
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>				
Dilution Water:	Freshwater Algal Medium				
	Light Intensity Measurements (lux)				
Test Day	No. 1	No. 2	No. 3	No. 4	No.5
0	3940	3880	4300	3900	3870
1	3910	4270	4440	4610	4010
2	4000	4290	4310	4540	4170
3	4120	4400	4350	4160	4240
4	4200	3990	3890	4070	4140
5	3910	4120	4180	4200	4340
6	4180	4200	4120	4200	4370
7	4300	4180	3950	4430	4080
8	4010	4230	4180	4230	4450
9	4150	4090	4240	4350	4270

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Table 4

pH Measurements

Sponsor:	3M Corporation	
Test Substance:	PFOS	
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>	
Dilution Water:	Freshwater Algal Medium	
Mean Measured Concentration (mg a.i./L)	pH Measurements	
	0 Hours ¹	96 Hours ²
Negative Control	7.5	8.1
5.5	7.4	8.2
11	7.5	8.2
21	7.5	8.4
44	7.4	8.3
86	7.4	7.8
179	7.4	7.5
169 (Abiotic)	--	7.4

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.

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Table 5

Mean Cell Densities and Percent Inhibition for Each 24-Hour Interval During the Test¹

Sponsor: 3M Corporation								
Test Substance: PFOS								
Test Organism: Freshwater Alga, <i>Selenastrum capricornutum</i>								
Dilution Water: Freshwater Algal Medium								
Mean Measured Concentration (mg a.i./L)	24 Hours		48 Hours		72 Hours		96 Hours	
	Mean Cell Density	Percent Inhibition	Mean Cell Density	Percent Inhibition	Mean Cell Density	Percent Inhibition	Mean Cell Density	Percent Inhibition
Negative Control	32,667	--	129,000	--	603,333	--	2,740,000	--
5.5	29,000	11	129,333	-0.26	635,000	-5.2	3,040,000	-11
11	29,333	10	121,000	6.2	615,000	-1.9	2,880,000	-5.1
21	28,000	14	133,667	-3.6	636,667	-5.5	3,240,000	-18
44	29,667	9.2	140,667	-9.0	535,000	11	3,080,000	-12
86	25,667	21	56,000	57	171,000 ²	72	626,667 ³	77
179	14,333	56	27,667	79	20,667 ²	97	33,667 ³	99

¹ Values calculated using SAS 6.12. Manual calculations may differ slightly

² Indicates a significant difference from the negative control at 72 hours using Dunnett's test ($p \leq 0.05$).

³ Indicates a significant difference from the negative control at 96 hours 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 Test¹

Sponsor:	3M Corporation							
Test Substance:	PFOS							
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>							
Dilution Water:	Freshwater Algal Medium							
Mean Measured Test Concentration (mg a.i./L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition
Negative Control	272,000	--	1,972,000	--	10,520,000	--	50,400,000	--
5.5	228,000	16	1,888,000	4.3	10,820,000	-2.9	54,680,000	-8.5
11	232,000	15	1,796,000	8.9	10,388,000	1.3	52,088,000	-3.3
21	216,000	21	1,916,000	2.8	10,920,000	-3.8	57,200,000	-13
44	236,000	13	2,040,000	-3.4	9,908,000	5.8	53,048,000	-5.3
86	188,000	31	928,000	53	3,412,000 ²	68	12,7444,000 ³	75
179	52,000	81	316,000	84	656,000 ²	94	1,068,000 ³	98
¹ Values calculated using SAS 6.12. Manual calculations may differ slightly								
² Indicates a significant difference from the negative control at 72 hours using Dunnett's test ($p \leq 0.05$).								
³ Indicates a significant difference from the negative control at 96 hours using Dunnett's test ($p \leq 0.05$).								

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Table 7

Mean Growth Rates and Percent Inhibition for Each 24-Hour Interval During the Test¹

Sponsor:	3M Corporation							
Test Substance:	PFOS							
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>							
Dilution Water:	Freshwater Algal Medium							
Mean Measured Test Concentration (mg a.i./L)	0 - 24 Hours		0 - 48 Hours		0 - 72 Hours		0 - 96 Hours	
	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition
Negative Control	0.0493	--	0.0533	--	0.0568	--	0.0585	--
5.5	0.0438	11	0.0533	0.0	0.0574	-1.1	0.0595	-1.9
11	0.0448	9.1	0.0518	2.7	0.0572	-0.68	0.0590	-0.84
21	0.0428	13	0.0540	-1.4	0.0572	-0.72	0.0602	-3.0
44	0.0452	8.3	0.0551	-3.3	0.0549	3.4	0.0597	-2.0
86	0.0373	24	0.0357	33	0.0392 ²	31	0.0429 ³	27
179	0.0138	72	0.0208	61	0.0100 ²	82	0.0124 ³	79

¹ Values calculated using SAS 6.12. Manual calculations may differ slightly

² Indicates a significant difference from the negative control at 72 hours using Dunnett's test ($p \leq 0.05$).

³ Indicates a significant difference from the negative control at 96 hours using Dunnett's test ($p \leq 0.05$).

Table 8

EC10, EC50 and EC90 Values Based on Cell Density Over the 96-Hour Exposure Period

Sponsor:		3M Corporation				
Test Substance:		PFOS				
Test Organism:		Freshwater Alga, <i>Selenastrum capricornutum</i>				
Dilution Water:		Freshwater Algal Medium				
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	--	163	74 and 191	Not Determined	--
48 Hours	Not Determined	--	81	72 and 90	Not Determined	--
72 Hours	37	<0.0 and 64	70	44 and 78	153	130 and 165
96 Hours	49	43 and 50	71	66 and 73	137	105 and 153

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Table 9

EC10, EC50 and EC90 Values Based on Area Under the Growth Curve Over the 96-Hour Exposure Period

Sponsor:	3M Corporation					
Test Substance:	PFOS					
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>					
Dilution Water:	Freshwater Algal Medium					
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	--	122	19 and 176	Not Determined	--
48 Hours	Not Determined	--	84	67 and 146	Not Determined	--
72 Hours	46	<0.0 and 56	74	55 and 82	165	145 and 176
96 Hours	49	40 and 50	71	67 and 74	145	125 and 155

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Table 10

EC10, EC50 and EC90 Values Based on Growth Rate Over the 96-Hour Exposure Period

Sponsor: 3M Corporation						
Test Substance: PFOS						
Test Organism: Freshwater Alga, <i>Selenastrum capricornutum</i>						
Dilution Water: Freshwater Algal Medium						
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	--	136	30 and 204	Not Determined	--
48 Hours	Not Determined	--	142	107 and 185	Not Determined	--
72 Hours	53	23 and 64	120	103 and 132	>179	-- ¹
96 Hours	59	54 and 63	126	115 and 138	>179	-- ¹
¹ Confidence limits could not be calculated with the data obtained.						

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Table 11

Cell Densities for the Recovery Phase

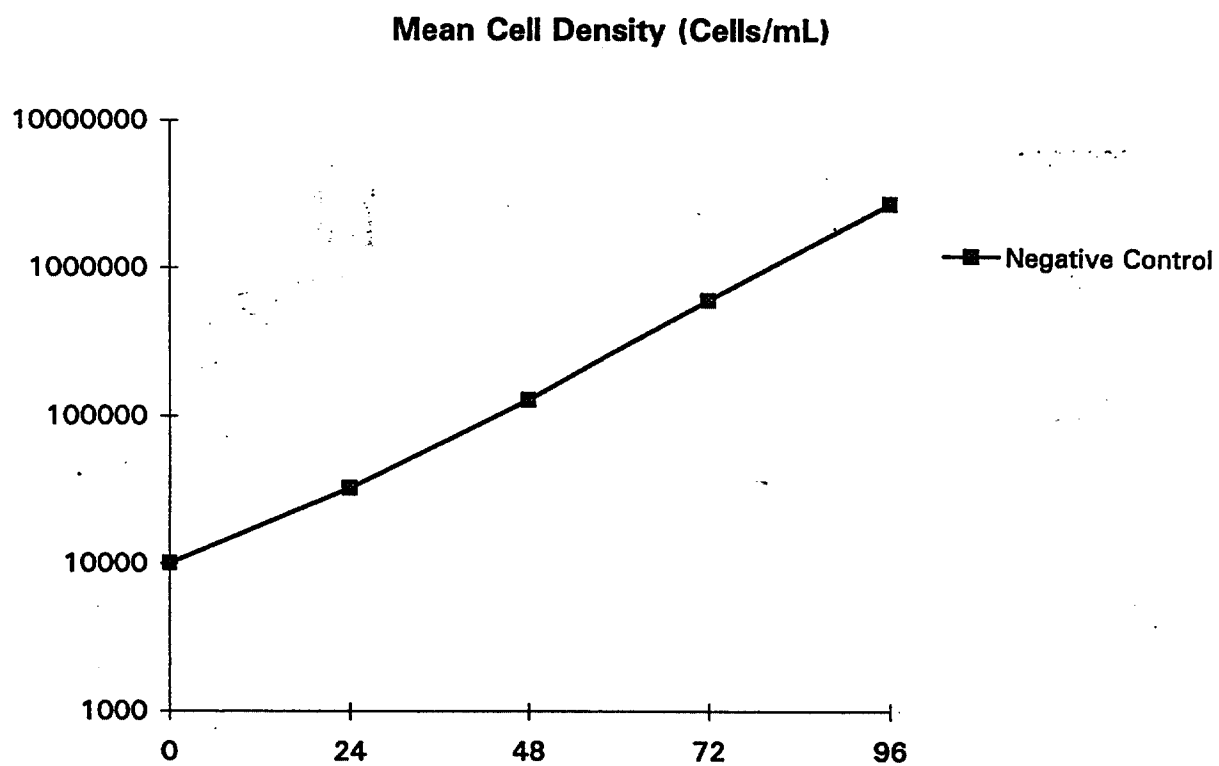
Sponsor:	3M Corporation		
Test Substance:	PFOS		
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>		
Dilution Water:	Freshwater Algal Medium		
Mean Measured Test Concentration (mg a.i./L)	Cell Densities (Cells/mL ²)		
	Day 0	Day 3	Day 5
Negative Control Rep. A	10,000	1,220,000	6,420,000
179 ¹	1,000	35,000	1,180,000
¹ The treatment group was diluted to a concentration of the test substance that theoretically would not inhibit growth.			
² Due to the method used to prepare recovery phase test solutions, initial cell densities were not equivalent throughout the treatment groups.			

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Figure 1. Negative Control Algal Growth, Expressed in Cell Density, During the 96-Hour Exposure.

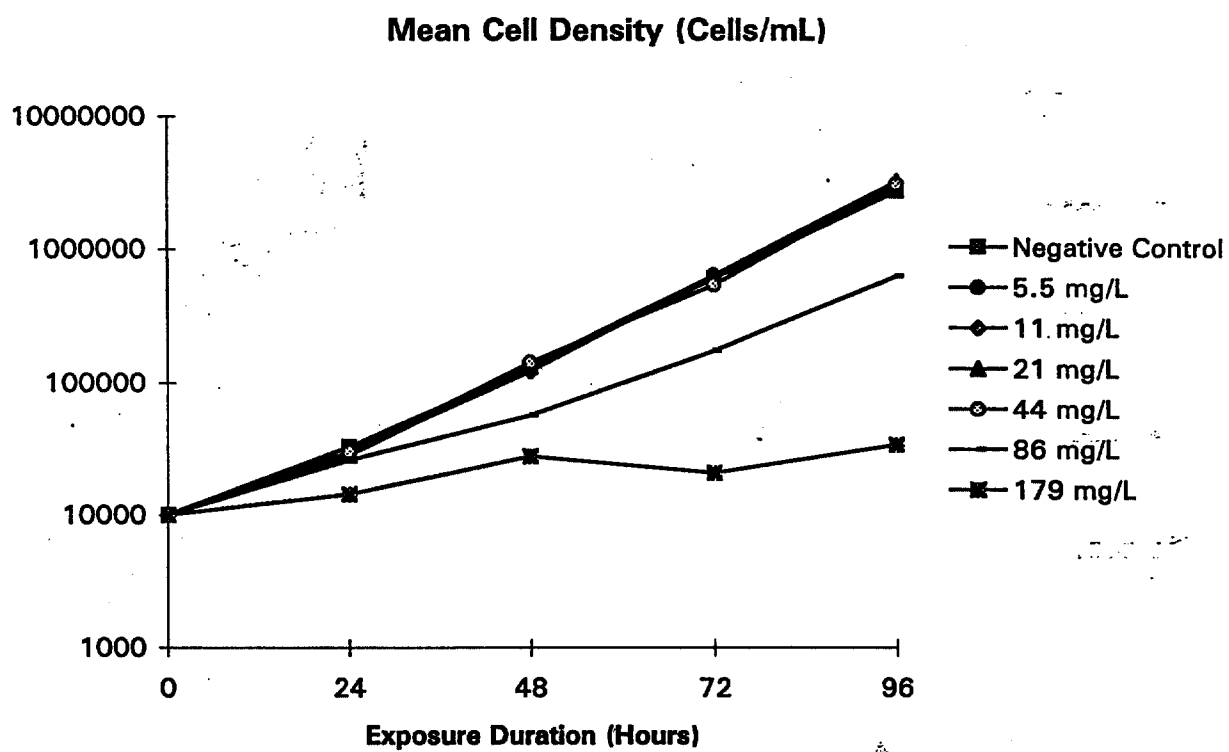


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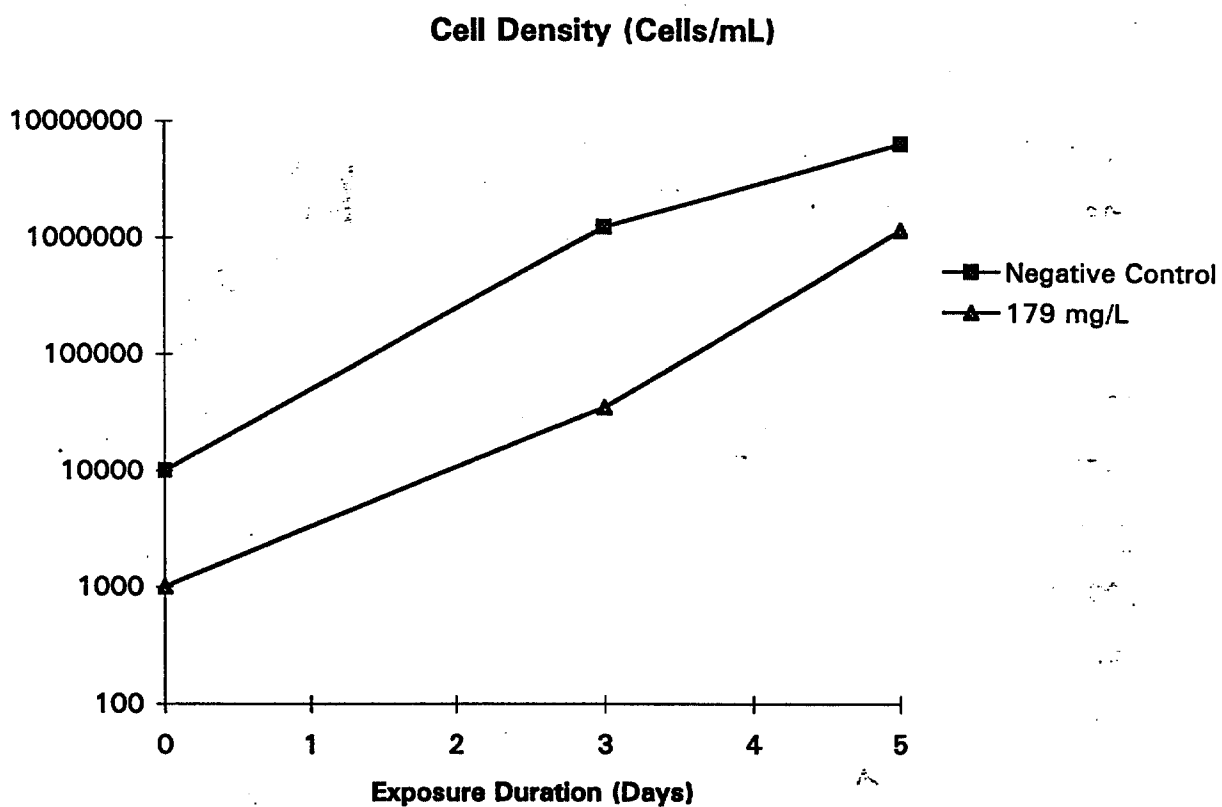
Figure 2. Concentration-Response Curve, Expressed in Cell Density



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Figure 3 - Recovery Phase Response Curve, Expressed in Cell Density



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APPENDIX I

Freshwater Algal Medium¹

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>
Dilution Water:	Freshwater Algal Medium

Compound	Nominal Concentration	
MgCl ₂ •6H ₂ O	12.16	mg/L
CaCl ₂ •2H ₂ O	4.40	mg/L
H ₃ BO ₃	0.1856	mg/L
MnCl ₂ •4H ₂ O	0.416	mg/L
ZnCl ₂	3.28	μg/L
FeCl ₃ •6H ₂ O	0.1598	mg/L
CoCl ₂ •6H ₂ O	1.428	μg/L
Na ₂ MoO ₄ •2H ₂ O	7.26	μg/L
CuCl ₂ •2H ₂ O	0.012	μg/L
Na ₂ EDTA•2H ₂ O	0.300	mg/L
NaNO ₃	25.50	mg/L
MgSO ₄ •7H ₂ O	14.70	mg/L
K ₂ HPO ₄	1.044	mg/L
NaHCO ₃	15.0	mg/L

¹ The pH was adjusted to 7.5 ± 0.1 using 10% HCl.

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	<	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.

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APPENDIX III

THE ANALYSIS OF PFOS IN FRESHWATER ALGAL MEDIUM

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-103A

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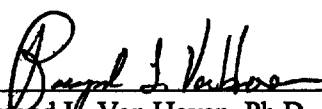
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-103A

PRINCIPAL INVESTIGATOR:



Raymond L. Van Hoven, Ph.D.
Scientist

4-24-00

DATE

MANAGEMENT:



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

4/24/00

DATE

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Introduction

Freshwater algal medium samples were collected from an acute toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the freshwater alga (*Selenastrum capricornutum*). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-103A. 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 on April 12, 15 and 16, 1999 and were analyzed on each sample receipt day.

Test Substance and Internal Standard

The test substance used for the analytical portion of this study was Wildlife International Ltd. identification number 4675. The test substance was used to prepare calibration standards 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 Validation for the Determination of PFOS in Freshwater, Saltwater, and Algal Media". 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 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 (100 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 0.00229 to 0.0457 mg a.i./L were analyzed with the samples. 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 0.115 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

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 chromatogram of a matrix blank is presented in Figure 5.

Freshwater algal medium was fortified at 0.915, 45.7 and 220 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 99.1%. A representative chromatogram of a matrix fortification is presented in Figure 6.

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Example Calculations

Sample number 454A-103A-2, nominal concentration of 5.7 mg a.i./L in freshwater algal medium.

Initial Volume: 0.100 mL

Final Volume: 25.0 mL

Dilution Factor: 250

PFOS Peak Area: 164427

Internal Standard Peak Area: 240533

Peak Area Ratio: 0.6836

Calibration curve equation.

Slope: 3.43926

Intercept: 0.03327

Curve is weighted (1/x).

$$\begin{aligned}
 \text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} &= \frac{(\text{Peak area ratio} - (\text{Y-intercept})) \times \text{I.S. Concentration}}{\text{Slope}} \\
 &= \frac{(0.6836 - 0.03327) \times 100 \mu\text{g a.i./L}}{3.43926} \\
 &= 18.9 \mu\text{g a.i./L}
 \end{aligned}$$

Note: I.S. = internal standard.

$$\begin{aligned}
 \text{PFOS (mg a.i./L) in sample} &= \frac{\text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} \times \text{Dilution Factor}}{1000 \mu\text{g/mg}} \\
 &= \frac{18.9 \times 250}{1000}
 \end{aligned}$$

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$$= 4.73 \text{ mg a.i./L}$$

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

Calculated recovery: 82.0%

Note: manual calculation may differ.

RESULTS

Sample Analysis

Freshwater algal medium samples were collected from the acute toxicity study with the freshwater alga (*Selenastrum capricornutum*) at test initiation, April 12, 1999 (Hour 0), on April 15, 1999 (Hour 72), and at test termination, April 16, 1999 (Hour 96). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 82.0 to 98.1% of the nominal concentrations. Samples collected at Hour 72 had a measured concentration range of 91.7 to 105% of nominal values. The Hour-72 abiotic sample was 81.9% of the nominal concentration. Samples collected at test termination (Hour 96) had a measured concentration range of 90.3 to 102% of nominal values. The Hour-96 abiotic sample was 103% of the nominal concentration (Table 4). A representative chromatogram of a test sample is shown in Figure 7.

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Table 1

Typical LC/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 (100 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	10.0 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 6.6 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 4.5 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-103A-)	Type	PFOS ¹ (mg a.i./L)
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ

¹ The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

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Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-103A-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered
	Fortified	Measured	
MAS-1	0.915	0.884	96.6
MAS-4	0.915	1.10	120
MAS-7	0.915	1.02	112
MAS-2	45.7	41.3	90.2
MAS-5	45.7	48.4	106
MAS-8	45.7	41.4	90.6
MAS-3A	220	210	95.2
MAS-3B	220	201	91.7
MAS-3C	220	209	95.0
MAS-6	220	221	101
MAS-9	220	202	91.9
Mean = 99.1			
Standard Deviation = 9.75			
CV = 9.84			
N = 11			
Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.			

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Table 4

Measured Concentrations of PFOS in Freshwater Algal Medium Samples from a
Freshwater Alga Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-103A-)	Sampling Time (Hours)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
0.0	1	0	< LOQ	--
	8	72	< LOQ	--
	15	96	< LOQ	--
5.7	2	0	4.73	82.0
	9	72	6.04	105
	16	96	5.84	101
11	3	0	10.7	89.8
	10	72	11.2	93.6
	17	96	12.1	102
23	4	0	19.8	86.2
	11	72	23.1	101
	18	96	20.7	90.3
46	5	0	42.7	93.4
	12	72	41.9	91.7
	19	96	46.3	101
91	6	0	83.3	91.0
	13	72	86.0	94.0
	20	96	88.3	96.5
183	7	0	179	98.1
	14	72	186	102
	21	96	172	94.0
183	14A	72	150	81.9
(Abiotic)	22	96	188	103

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

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**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.



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 LC/MS analysis.

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

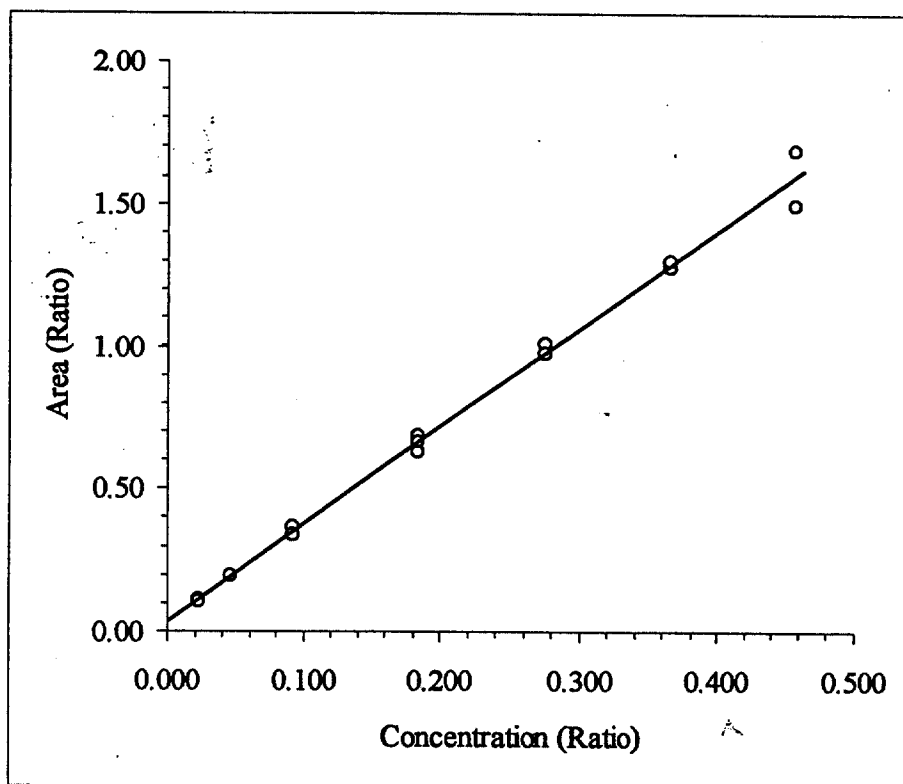


Figure 2. A typical calibration curve for PFOS. Slope = 3.43926; Intercept = 0.03327; $r = 0.9986$.
Curve is weighted ($1/x$).

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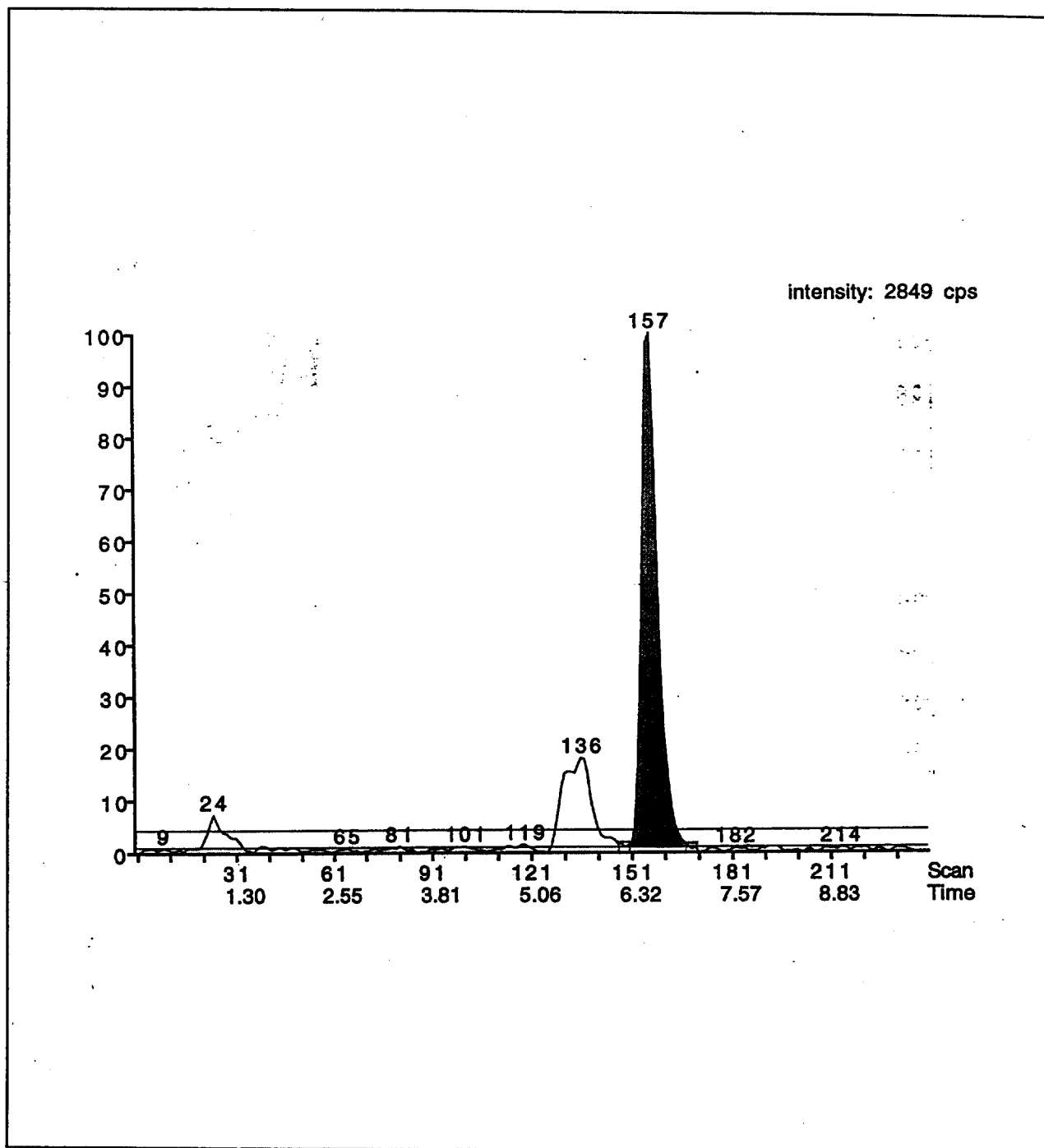


Figure 3. A representative ion chromatogram of a low-level (0.00229 mg a.i./L) PFOS standard.

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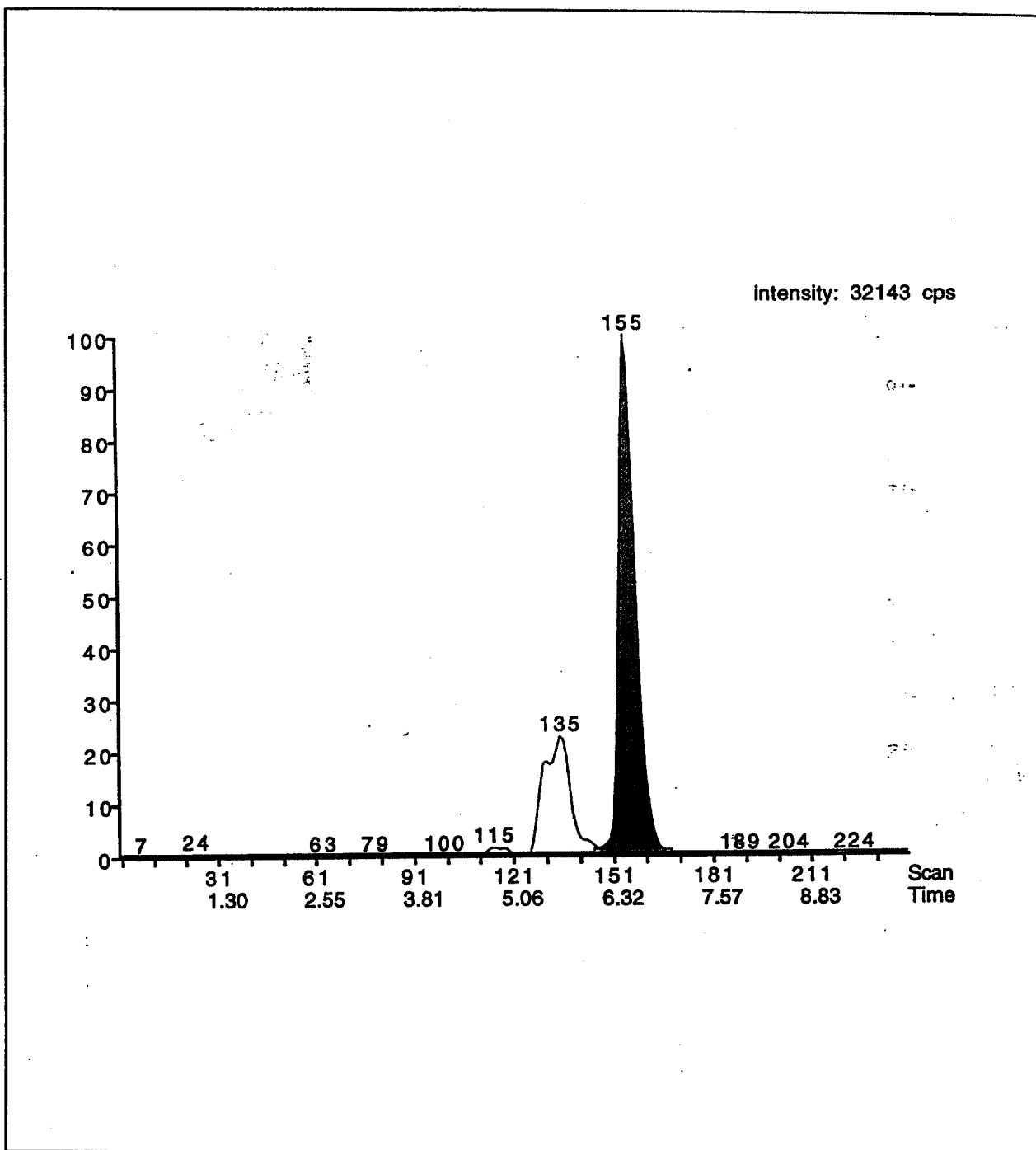


Figure 4. A representative ion chromatogram of a high-level (0.0457 mg a.i./L) PFOS standard.

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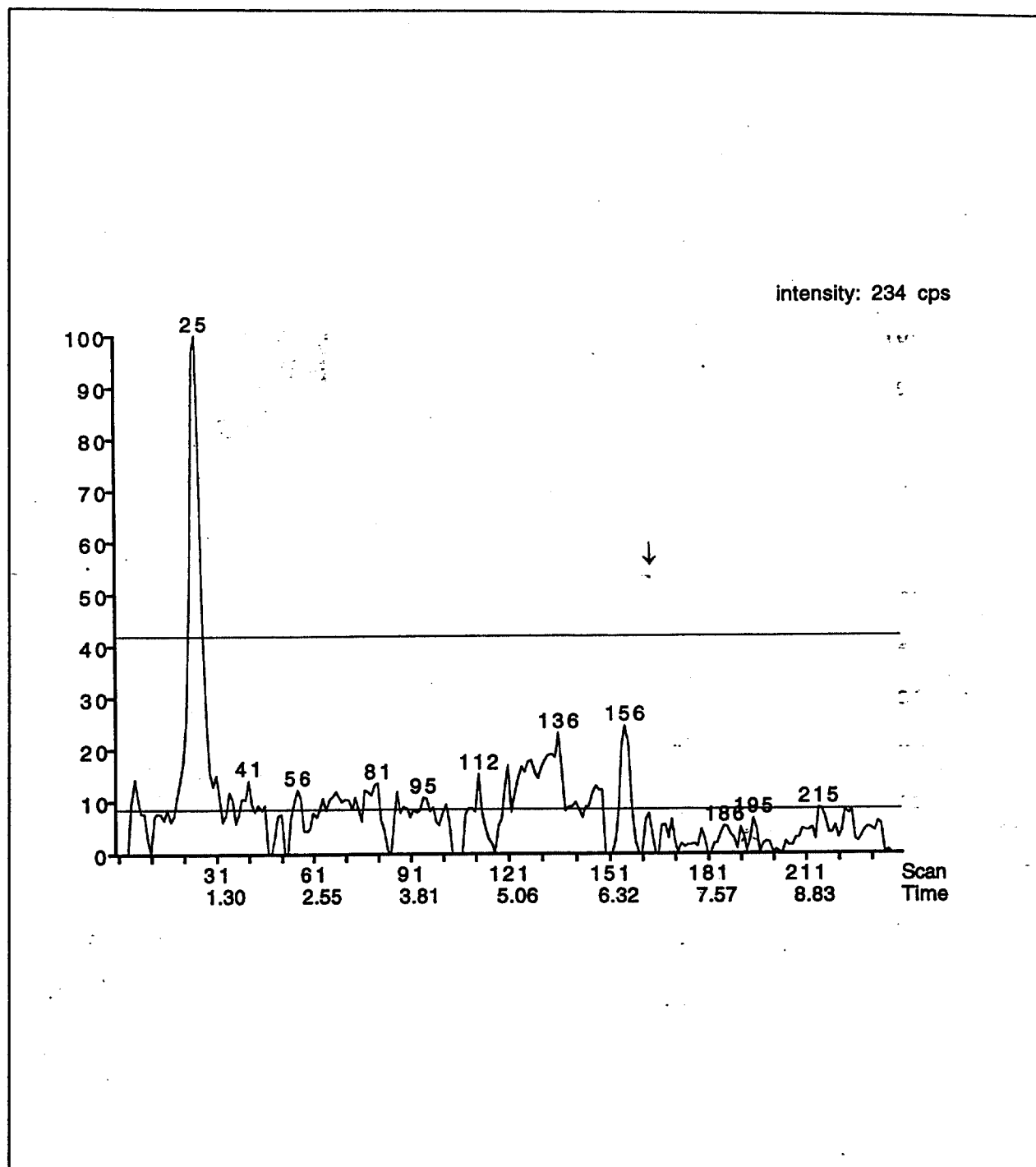


Figure 5. A representative chromatogram of a matrix blank sample (454A-103A-MAB-1).
The arrow indicates the retention time of PFOS.

000655

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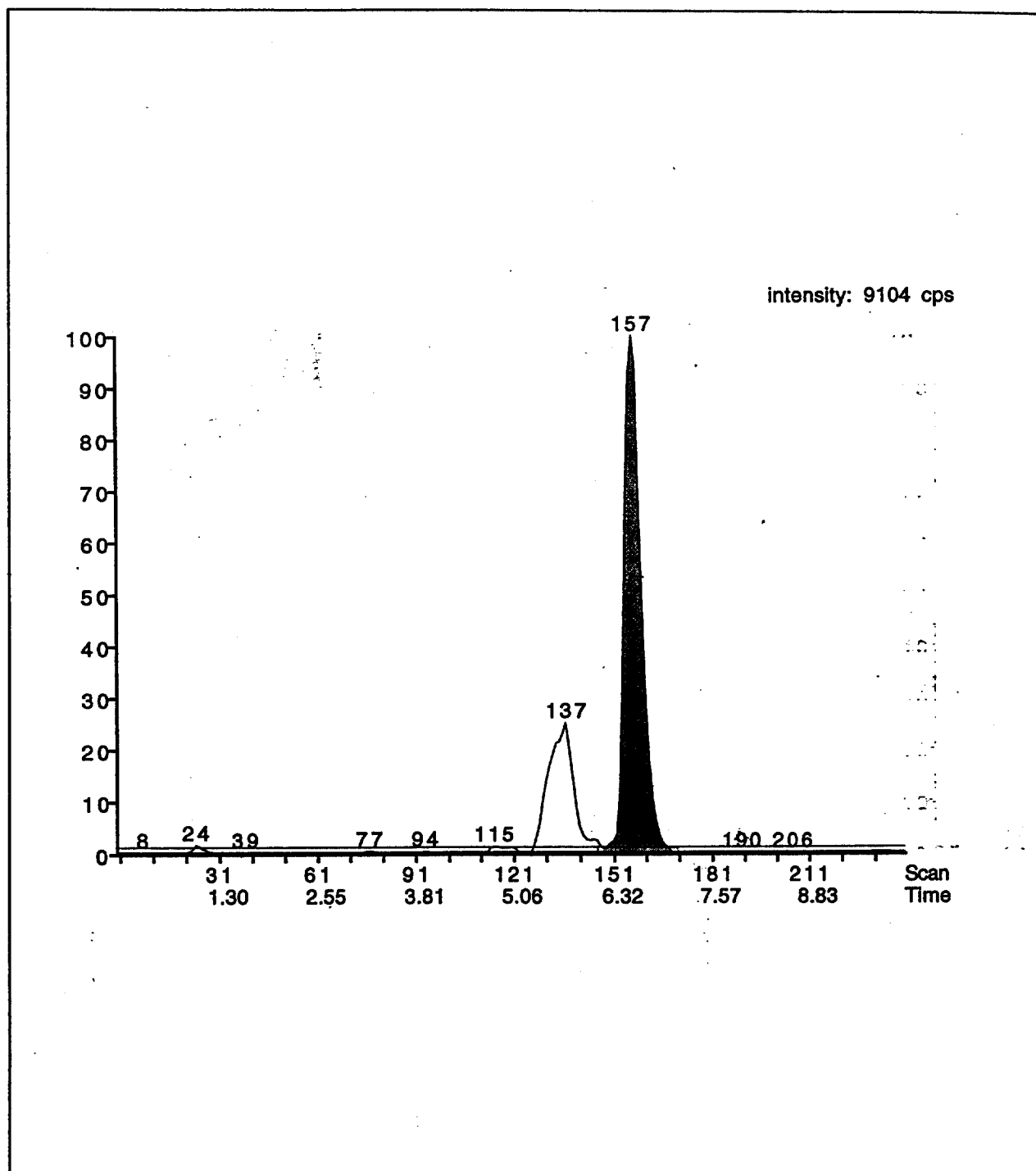


Figure 6. A representative chromatogram of a matrix fortification sample (454A-103A-MAS-1).

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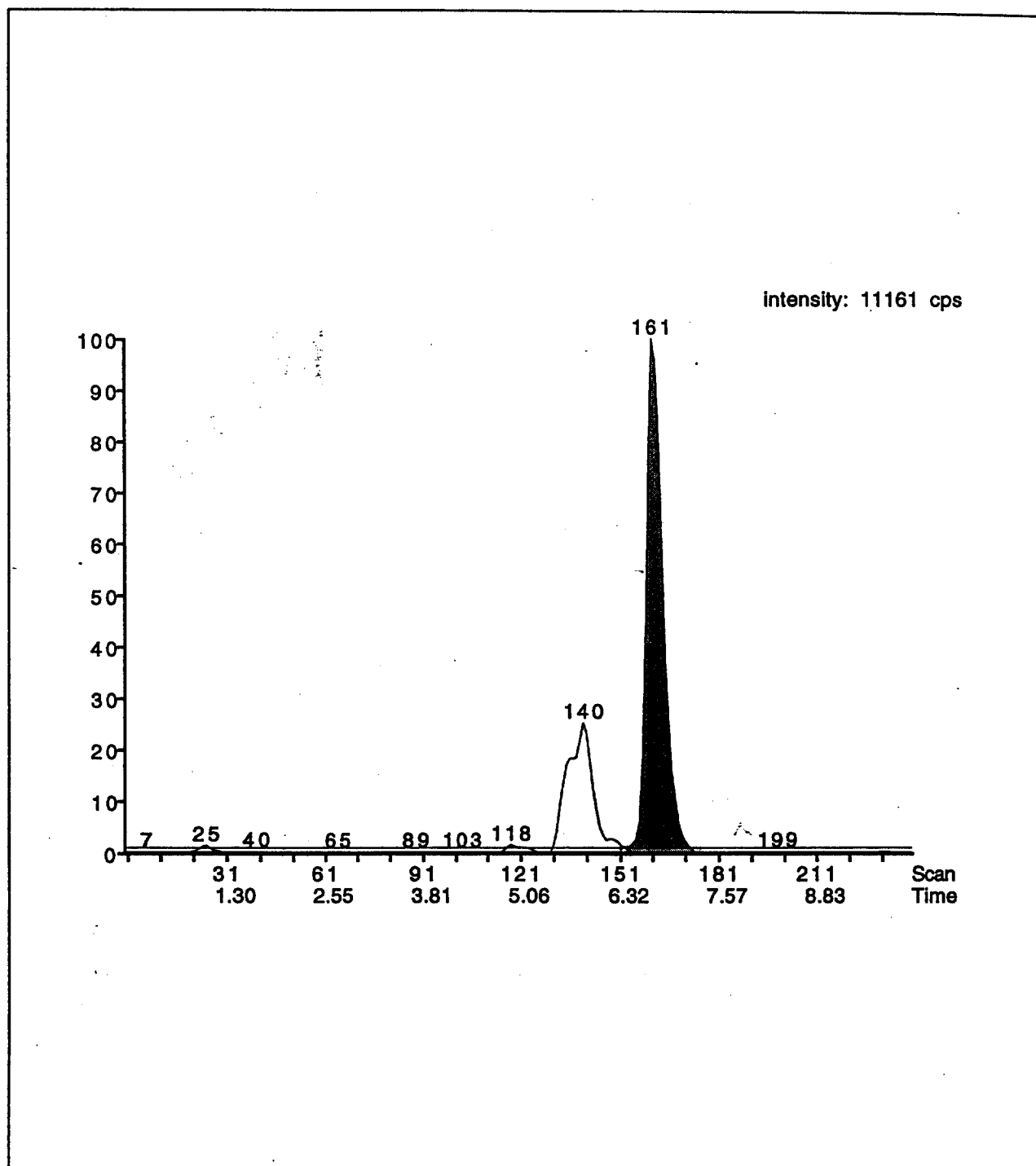


Figure 7. A representative chromatogram of a test sample (454A-103A-2).

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APPENDIX IV

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

Sponsor: 3M Corporation					
Test Substance: PFOS					
Test Organism: Freshwater Alga, <i>Selenastrum capricornutum</i>					
Dilution Water: Freshwater Algal Medium					
Mean Measured Concentration (mg a.i./L)	Replicate	Cell Densities (Cells/mL) ¹			
		24 Hours	48 Hours	72 Hours	96 Hours
Negative Control	A	34,000	132,000	710,000	2,720,000
	B	33,000	130,000	605,000	2,860,000
	C	31,000	125,000	495,000	2,640,000
5.5	A	29,000	136,000	780,000	3,000,000
	B	23,000	118,000	500,000	3,180,000
	C	35,000	134,000	625,000	2,940,000
11	A	30,000	105,000	560,000	2,680,000
	B	27,000	124,000	660,000	2,740,000
	C	31,000	134,000	625,000	3,220,000
21	A	30,000	141,000	860,000	3,340,000
	B	26,000	132,000	450,000	3,080,000
	C	28,000	128,000	600,000	3,300,000
44	A	30,000	136,000	515,000	2,800,000
	B	32,000	151,000	700,000	3,020,000
	C	27,000	135,000	390,000	3,420,000
86	A	37,000	65,000	215,000	650,000
	B	18,000	57,000	136,000	760,000
	C	22,000	46,000	162,000	470,000
179	A	15,000	35,000	21,000	45,000
	B	18,000	22,000	18,000	30,000
	C	10,000	26,000	23,000	26,000

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).

000658

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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 Alga, <i>Selenastrum capricornutum</i>					
Dilution Water: Freshwater Algal Medium					
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	288,000	2,040,000	11,904,000	52,824,000
	B	276,000	1,992,000	10,572,000	51,912,000
	C	252,000	1,884,000	9,084,000	46,464,000
5.5	A	228,000	1,968,000	12,720,000	57,840,000
	B	156,000	1,608,000	8,784,000	52,704,000
	C	300,000	2,088,000	10,956,000	53,496,000
11	A	240,000	1,620,000	9,360,000	48,000,000
	B	204,000	1,776,000	10,944,000	51,504,000
	C	252,000	1,992,000	10,860,000	56,760,000
21	A	240,000	2,052,000	13,824,000	63,984,000
	B	192,000	1,848,000	8,592,000	50,712,000
	C	216,000	1,848,000	10,344,000	56,904,000
44	A	240,000	1,992,000	9,564,000	49,104,000
	B	264,000	2,220,000	12,192,000	56,592,000
	C	204,000	1,908,000	7,968,000	53,448,000
86	A	324,000	1,308,000	4,428,000	14,568,000
	B	96,000	756,000	2,832,000	13,344,000
	C	144,000	720,000	2,976,000	10,320,000
179	A	60,000	420,000	852,000	1,404,000
	B	96,000	336,000	576,000	912,000
	C	0	192,000	540,000	888,000

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APPENDIX VI

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

Sponsor:		3M Corporation			
Test Substance:		PFOS			
Test Organism:		Freshwater Alga, <i>Selenastrum capricornutum</i>			
Dilution Water:		Freshwater Algal Medium			
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	0.0510	0.0538	0.0592	0.0584
	B	0.0497	0.0534	0.0570	0.0589
	C	0.0471	0.0526	0.0542	0.0581
5.5	A	0.0444	0.0544	0.0605	0.0594
	B	0.0347	0.0514	0.0543	0.0600
	C	0.0522	0.0541	0.0574	0.0592
11	A	0.0458	0.0490	0.0559	0.0582
	B	0.0414	0.0525	0.0582	0.0585
	C	0.0471	0.0541	0.0574	0.0602
21	A	0.0458	0.0551	0.0619	0.0605
	B	0.0398	0.0538	0.0529	0.0597
	C	0.0429	0.0531	0.0569	0.0604
44	A	0.0458	0.0544	0.0547	0.0587
	B	0.0485	0.0566	0.0590	0.0595
	C	0.0414	0.0542	0.0509	0.0608
86	A	0.0545	0.0390	0.0426	0.0435
	B	0.0245	0.0363	0.0363	0.0451
	C	0.0329	0.0318	0.0387	0.0401
179	A	0.0169	0.0261	0.0103	0.0157
	B	0.0245	0.0164	0.0082	0.0114
	C	0.0000	0.0199	0.0116	0.0010

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AMENDED

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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 number.
2. The protocol was amended to specify the analytical method.
3. The protocol was amended to repeat the test with a higher test concentration.

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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., Manager, Analytical Chemistry
3. Raymond L. VanHoven, Ph.D., Scientist
4. Kurt R. Drott, Senior Biologist
5. Mark A. Mank, Laboratory Supervisor

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APPENDIX IX

Report Amendment

1. Original Report: Pages 1-4, 6 and 36
Amendment: The pages were changed to include the amended report date, revised page numbers, and new signatures and dates due to the addition of the report amendment as Appendix IX.
Reason: To reflect the issuing of an amended report.
2. Original Report: Page 2
Amendment: The compliance statement was revised.
Reason: To clarify how the test substance was characterized.
3. Original Report: Page 10
Amendment: Information provided by the Sponsor reflecting the reanalysis of the test substance, including the reanalysis date and the purity, was added to the Test Substance section.
Reason: To reflect the current test substance information provided by the Sponsor.
4. Original Report: Entire report
Amendment: All test substance concentrations were changed to reflect the purity of the test substance as determined by the Sponsor in a reanalysis of the test substance (FC-95, Lot 217). Test concentrations originally were based on the reported purity of 98.9%. The certificate of analysis dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations, including nominal concentrations, measured concentrations, EC values, and NOAEC values were recalculated and reported as mg a.i./L based on the 90.49% purity.
Reason: To report the results of the test based on the test substance purity of 90.49% at the request of the Sponsor.

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AMENDED

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA
(*Selenastrum capricornutum*)

PROTOCOL NO.: 454/110998/SEL-96H1/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-103

EFFECTIVE DATE: January 4, 1999

AMENDMENT: Page 2

Add: Experimental Start Date: 2/19/99

Experimental Termination Date: 2/23/99

Test Concentrations: Negative Control, 6.3, 13, 25, 50 and 100 mg a.i./L

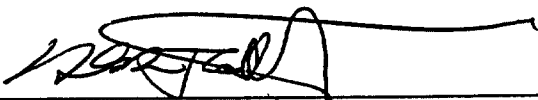
Test Substance No.: 4675

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

AMENDMENT: APPENDIX II, Page 16

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

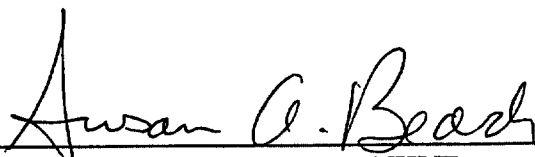
REASON: To add the analytical method to be used in the study.


STUDY DIRECTOR

2/19/99
DATE


LABORATORY MANAGEMENT

2/19/99
DATE


SPONSOR'S REPRESENTATIVE

2/16/99
DATE

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA
(*Selenastrum capricornutum*)

PROTOCOL NO.: 454/110998/SEL-96H1/SUB454

AMENDMENT NO.: 2

SPONSOR: 3M Corporation

PROJECT NO.: 454A-103

EFFECTIVE DATE: April 8, 1999

AMENDMENT: Page 2

Change: Experimental Start Date: 2/19/99
Experimental Termination Date: 2/23/99
Test Concentrations: Negative Control, 6.3, 13, 25, 50 and 100 mg a.i./L

To: Experimental Start Date: 4/12/99
Experimental Termination Date: 4/16/99
Test Concentrations: Negative Control, 6.3, 13, 25, 50, 100 and 200 mg a.i./L

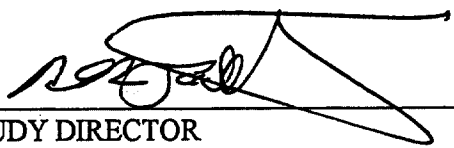
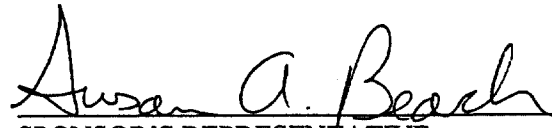
REASON: The test will be repeated because cell counts were not made with a hemacytometer and a microscope. An additional test concentration was added in an attempt to obtain EC90 values.

AMENDMENT: EXPERIMENTAL DESIGN, Page 3

Change: Target concentrations will not exceed 100 mg/L or the solubility limit of the test substance in water.

To: Target concentrations will not exceed 1000 mg/L or the solubility limit of the test substance in water.

REASON: The new nominal concentrations exceed 100 mg a.i./L.


STUDY DIRECTOR4/12/99
DATE
LABORATORY MANAGEMENT4/12/99
DATE
SPONSOR'S REPRESENTATIVE4/29/99
DATE

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PROTOCOL

PFOS: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Selenastrum capricornutum*)

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

OECD Guideline 201

3M Lab Request No. U2723

Submitted to

3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133



WILDLIFE INTERNATIONAL LTD.



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

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November 9, 1998

PROTOCOL NO.: 454/110998/SEL-96H1/SUB454

3M LAB REQUEST NO. U2723

PFOS: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Selenastrum capricornutum*)

SPONSOR:

3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133

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: _____

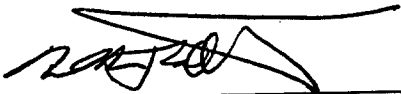
Experimental
Termination Date: _____

Project No.: 454A-103

Test Concentrations: _____

Test Substance No.: _____ Reference Substance No. (if applicable): _____

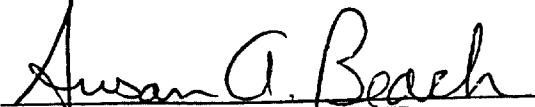
PROTOCOL APPROVAL


STUDY DIRECTOR

12/4/98
DATE


LABORATORY MANAGEMENT

12/4/98
DATE


SPONSOR'S REPRESENTATIVE

12/1/98
DATE

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INTRODUCTION

Wildlife International Ltd. will conduct a 96-hour toxicity test with the freshwater alga, *Selenastrum capricornutum*, 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 850.5400 (1) and OECD Guideline for Testing of Chemicals, 201: *Alga, Growth Inhibition Test* (2). 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 toxicity of a test substance to the freshwater green alga, *Selenastrum capricornutum*, during a 96-hour exposure period.

EXPERIMENTAL DESIGN

The green alga, *Selenastrum capricornutum*, will be exposed to a geometric series of at least five test concentrations and a negative (culture medium) control for 96 hours. Target concentrations will not exceed 100 mg/L or the solubility limit of the test substance in water. Generally, the nominal concentration of each test substance used in the definitive test will be at least 50% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate.

Test solutions will be inoculated with 10,000 cells/mL. Three "biological" replicates per experimental group will be prepared for evaluating cell densities. One or more additional "analytical" replicates will be included in the test, as needed, to provide test solution for concentration verification on Day 3 of the exposure. An abiotic replicate at the highest concentration will be included in the test and will be sampled on Day 3 and Day 4. In order to control bias, the position of the flasks will be determined by indiscriminate draw daily during the exposure period. No other potential sources of bias are expected to affect the results of the study.

The response of the algae will be measured in terms of cell density, biomass expressed as area under the growth curve, and growth rate. An EC50 (i.e., the theoretical test concentration that produces a 50% reduction in the measured parameter) value for cell density will be calculated for each 24 hour interval. EC10, EC50 and EC90 values for cell density, biomass (E_0C) and growth rate (E_0C) will be

calculated, if possible, at the 72 and 96 hour intervals. The no observed adverse effect concentration (NOAEC), which is the highest test concentration that induces no adverse inhibitory effect on growth, will be determined relative to each parameter at 72 and 96 hours based upon evaluation of the statistical results and the dose-response pattern. At the end of the 96-hour exposure, algistatic effects will be differentiated from algicidal effects in those treatments which are maximally inhibited.

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 and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Solutions

The test substance will be administered to *Selenastrum* in an aqueous medium. This route of administration was selected because it represents the most likely route of exposure to algae, which exists in aquatic environments.

Test solutions will be prepared by diluting one or more stock solutions with algal culture medium to achieve the target concentrations. Stocks will be prepared in algal culture medium. All weights, volumes and dilutions used to prepare the test solutions will be documented in the raw data.

Test Organism

The freshwater green alga, *Selenastrum capricornutum*, will be used in this test. This species is representative of an important group of algae and was selected for use in the test based upon past use and ease of handling in the laboratory. Stock cultures, obtained from the Culture Collection of Algae at the University of Texas at Austin or another supplier, will be maintained in culture medium at Wildlife International Ltd. for a minimum of two weeks prior to use in a toxicity test. Algae used in toxicity tests

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will be in exponential growth phase, which is defined as the period of growth when algal cells are dividing at a constant rate.

Just prior to beginning the test, an inoculum of the stock culture will be prepared so that each milliliter of inoculum contains enough cells to provide an initial cell density of approximately 10,000 cells/mL in each replicate.

Culture Medium

Culture medium prepared according to Wildlife International Ltd. Standard Operating Procedures will be used as dilution water. The concentrations of the components in the medium are presented in Table 1 (3). Stock nutrient solutions will be prepared by adding reagent-grade chemicals to Wildlife International Ltd. well water purified by reverse-osmosis. Appropriate volumes of the stock nutrient solutions will then be diluted with purified well water to prepare the medium. The medium will be filter sterilized (0.2 μ m or autoclaved) prior to use. The final pH of the medium should be 7.5 ± 0.1 . 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 analyses will be summarized in the final report.

Specifications for acceptable levels of contaminants have not been established for culture medium. However, there are no levels of contaminants reasonably expected to be present in the medium that are considered to interfere with the purpose or conduct of the study.

Test Apparatus

Test chambers will be sterile, 250-mL polycarbonate Erlenmeyer flasks plugged with foam stoppers. Each test chamber will hold 100 mL of test solution. Test chambers will be indiscriminately positioned on a mechanical shaker table in an environmental chamber and will be shaken continuously at approximately 100 rpm. Test chambers will be labeled with the project number, test concentration, and replicate.

Environmental Conditions

Test flasks will be held at $24 \pm 2^\circ\text{C}$ under continuous fluorescent lighting (e.g., cool white light tubes) at an intensity of approximately $4300 \pm 10\%$ lux. Light intensity will be measured at five locations on the shaker table daily during the test. Temperature will be measured twice daily in the environmental chamber using a liquid-in-glass thermometer.

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The pH of each treatment and the control will be measured at test initiation and termination using a Fisher Accumet Model 915 pH meter or equivalent. Samples will be collected from the batch of test solution prepared at test initiation for each treatment and control group for the measurement of pH. At test termination, pH will be measured in pooled test solution samples collected from the three replicates of each treatment and control group.

Biological Measurements

Cell densities will be monitored during the test by conducting cell counts using a hemacytometer and a microscope. One sample will be collected from each replicate of the treatment and control groups at approximately 24 hour intervals during the exposure period. The samples will be evaluated immediately. Each sample will be diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. The cell density of the sample will be calculated based on the mean number of cells per grid.

Samples of test solutions will be collected from each replicate per treatment and control group at the end of the test. Each replicate sample will be examined microscopically for atypical cell morphology (e.g., changes in shape, size or color). Growth of cells in the flasks also will be assessed for aggregations or flocculations of cells and adherence of the cells to the test chamber.

At the end of the test, algistatic effects, which result in the inhibition of cell growth, will be differentiated from algicidal effects, which result in the death of cells. Aliquots (0.5 mL) of test solutions will be taken from each replicate test chamber where growth is maximally inhibited. Maximally inhibited treatments are those in which it cannot be visually determined if live algal cells are present (e.g. absence of color to the test solution). These aliquots will be combined and diluted with algal medium to a concentration of the test substance that theoretically should not affect growth. A negative control will be prepared by diluting a 0.5-mL aliquot from one negative control replicate to 100 mL with algal medium. Growth of these subcultures will be monitored for up to 9 days to determine whether inhibition of growth observed during the test is reversible. Samples will be collected for cell counts at recovery phase initiation, termination and at approximately 3-day intervals between initiation and termination. The recovery phase may be terminated once algal growth is sufficient to indicate that the algal cells have fully recovered from effects due to the test substance. This will be determined through visual examination of the recovery test solutions.

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Sampling for Analytical Measurements

Samples of the exposure solutions will be collected on Days 0, 3 and 4 and analyzed for test substance concentrations. Day 0 samples will be taken from each batch of test solution prior to their distribution among the replicate test chambers. Day 3 samples will be collected from the extra replicate test chamber(s) included in the test to provide sample for that analysis and from the abiotic replicates. If more than one replicate is included for the Day 3 sample analysis, then the solutions from each replicate will be composited to provide the sample for that treatment. Day 4 samples will be composited solutions from the three replicates remaining at the end of the test.

Samples collected on Days 3 and 4 may be centrifuged or filtered to remove algal cells prior to analysis. Samples will be analyzed immediately or placed in an appropriate storage container (e.g., polypropylene or polyethylene bottle) and stored until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 3	Day 4
Control	1	1	1
Solvent Control (if needed)	1	1	1
Level 1-Low Concentration	1	1	1
Level 2	1	1	1
Level 3	1	1	1
Level 4	1	1	1
Level 5-High Concentration	1	1	1
Totals	7	7	7

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.

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Analytical Chemistry

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. Modifications made to the analytical method will be documented in the raw data and described in the final report.

Statistical Analyses

Biomass expressed as area under the growth curve will be calculated for the treatment and control groups using the following formula:

$$A = ((N_1 - N_0)/2)(t_1) + ((N_1 + N_2 - 2N_0)/2)(t_2 - t_1) + \dots + ((N_{n-1} + N_n - 2N_0)/2)(t_n - t_{n-1})$$

Where:

A = Area under the growth curve
N₀ = Measured number of cells/mL at t₀
N₁ = Measured number of cells/mL at t₁
N₂ = Measured number of cells/mL at t₂
N_n = Measured number of cells/mL at t_n
t₁ = Time of first measurement after beginning of test (hours)
t₂ = Time of second measurement after beginning of test (hours)
t_n = Time of nth measurement after beginning of test (hours)

Growth rates (μ) will be calculated for the treatment and control groups using the following formula:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

μ = Average specific growth rate
N₀ = Measured number of cells/mL at t₀
N_n = Measured number of cells/mL at t_n
t₀ = Time of beginning of test (hours)
t_n = Time of nth measurement after beginning of test (hours)

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Percent inhibition will be calculated for each treatment group based upon mean cell density, biomass and growth rate using the following formula:

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

EC values will be determined, when possible, using linear interpolation or other suitable techniques with treatment response (i.e. cell densities, biomass and growth rate) and exposure concentration data for each 24-hour interval of the test.

A no observed adverse effect concentration (NOAEC) will be selected based on an evaluation of the dose-response pattern and the results of the statistical analysis using individual replicate values of the cell density, biomass and growth rate. Statistically significant differences between the control and the treatment groups will be identified using analysis of variance (ANOVA) and a test to compare treatment mean responses to the control response (e.g., Dunnett's test or Bonferroni's t-test). Data will be assessed for normality and homogeneity of variances prior to performing the ANOVA. Transformations will be used to correct any condition of non-normality or unequal error variances, if possible. If a solvent control is used in addition to the negative control, their responses will be compared using Student's t-test. Data from the two control groups will be pooled for treatment level analyses if no statistical differences exist. Otherwise, all comparisons will be made to the solvent control group. Statistical comparisons for the ANOVA and the comparison of mean responses will be carried out using TOXSTAT® V3.5 software (4) or equivalent.

RECORDS TO BE MAINTAINED

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

1. Copy of signed protocol.
2. Identification and characterization of the test substance, if provided by Sponsor.
3. Dates of initiation and termination of the test.
4. Source of algae.
5. Culture conditions.

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6. Growth measurements.
7. Calculation and preparation of test concentrations.
8. Observations.
9. The methods used to analyze test substance concentrations and the results of analytical measurements.
10. Statistical calculations.
11. Test conditions and physical/chemical measurements.
12. 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, and the definitive experimental start and termination dates.
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 all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information 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.
8. A description of the test organisms, including the source, scientific name, age or life stage, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. 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.
11. A description of circumstances that may have affected the quality or integrity of the data.

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12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration response curve.
14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
16. The location where raw data and final report are to be stored.
17. 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.
18. 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 amendment, and will be signed 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 (OCDE/GD (92) 32, Environment Monograph No. 45); 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 850.5400: Algal Toxicity, Tiers I and II.
- 2 OECD Guideline for Testing of Chemicals. 1984. 201: *Alga, Growth Inhibition Test*.
- 3 ASTM Standard Guide 1218-90E. 1990. *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae*.
- 4 West, Inc. and D. D. Gulley. 1996. *TOXSTAT Release 3.5*. Western EcoSystems Technology, Inc. Cheyenne, Wyoming.

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TABLE 1
FRESHWATER ALGAL MEDIUM CONSTITUENTS

Component	Nominal Concentration
MgCl ₂ •6H ₂ O	12.16 mg/L
CaCl ₂ •2H ₂ O	4.40 mg/L
H ₃ BO ₃	0.1856 mg/L
MnCl ₂ •4H ₂ O	0.416 mg/L
ZnCl ₂	3.28 µg/L
FeCl ₃ •6H ₂ O	0.1598 mg/L
CoCl ₂ •6H ₂ O	1.428 µg/L
Na ₂ MoO ₄ •2H ₂ O	7.26 µg/L
CuCl ₂ •2H ₂ O	0.012 µg/L
Na ₂ EDTA•2H ₂ O	0.300 mg/L
NaNO ₃	25.50 mg/L
MgSO ₄ •7H ₂ O	14.70 mg/L
K ₂ HPO ₄	1.044 mg/L
NaHCO ₃	15.00 mg/L

The pH of the medium will be adjusted, as necessary, to 7.5 ± 0.1 using 0.1 N NaOH or 10% HCl.

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APPENDIX II

Analytical Method to be Provided by Sponsor

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