13) OECD 201-OPPTS 850.5400, Algal toxicity- 96-Hour toxicity test with the freshwater alga, 454A-129

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

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-129

3M Environmental Lab Project No. E00-1429

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

AUTHORS:

Kurt R. Drottar
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: October 6, 2000

STUDY COMPLETION DATE: March 20, 2001

Submitted to

3M Corporation
Environmental laboratory
Building 2-3E-09
935 Bush Avenue
St. Paul, Minnesota 55144

Wildlife International, Ltd.

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

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

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

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-129

STUDY COMPLETION: March 20, 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 compliance with Good Laboratory Practices prior to its use in the study. However, subsequent GLP compliant characterization resulted in a purity similar to the original characterization purity.

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. Drottar
Senior Biologist

3/20/01
DATE

SPONSOR:

3/23/01
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	January 22, 2001	January 22, 2001	January 22, 2001
Sample Preparation	January 22, 2001	January 22, 2001	January 24, 2001
Biological Data and Draft Report	February 15 and 16, 2001	February 16, 2001	February 19, 2001
Analytical Data and Draft Report	February 15 and 16, 2001	February 16, 2001	February 16, 2001
Final Report	March 19, 2001	March 19, 2001	March 20, 2001

Marshall T. Hynson
Quality Assurance Program Supervisor

3/20/2001
DATE

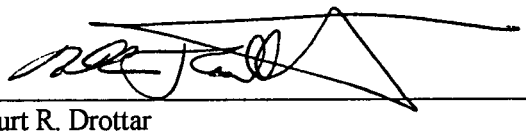
REPORT APPROVAL

SPONSOR: 3M Corporation

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

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-129

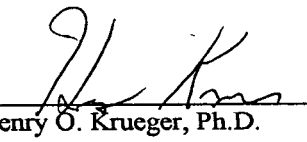
STUDY DIRECTOR:



Kurt R. Drottar
Senior Biologist

3/20/01
DATE

MANAGEMENT:



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

3/20/01
DATE

TABLE OF CONTENTS

TABLES

TABLE OF CONTENTS
-Continued-

FIGURES

APPENDICES

SANITIZED

DEC 0 9 2003

SUMMARY

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-129
TEST SUBSTANCE:	Perfluorobutanesulfonate, Potassium Salt (PFBS)
STUDY:	PFBS: A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L
TEST DATES:	Experimental Start – January 22, 2001 Exposure Termination – January 26, 2001 Experimental Termination – February 5, 2001
LENGTH OF TEST:	96 Hour Exposure, 6 Day Recovery

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

CELL DENSITY 72-HOUR EC50: 95% CONFIDENCE LIMITS:	 1469 mg a.i./L 81 and 2812 mg a.i./L
96-HOUR EC10: 95% CONFIDENCE LIMITS:	528 mg a.i./L 375 and 1895 mg a.i./L
96-HOUR EC50: 95% CONFIDENCE LIMITS:	2347 mg a.i./L 2018 and 2707 mg a.i./L
96-HOUR EC90: 95% CONFIDENCE LIMITS:	7390 mg a.i./L 7270 and 7500 mg a.i./L
72-HOUR NOAEC:	<285 mg a.i./L
96-HOUR NOAEC:	1077 mg a.i./L

SUMMARY (Continued)

AREA UNDER THE GROWTH CURVE	
72-HOUR EC50:	1590 mg a.i./L
95% CONFIDENCE LIMITS:	328 and 2373 mg a.i./L
96-HOUR EC10:	385 mg a.i./L
95% CONFIDENCE LIMITS:	191 and 951 mg a.i./L
96-HOUR EC50:	2146 mg a.i./L
95% CONFIDENCE LIMITS:	1748 and 2706 mg a.i./L
96-HOUR EC90:	7294 mg a.i./L
95% CONFIDENCE LIMITS:	6888 and 7673 mg a.i./L
72-HOUR NOAEC:	<285 mg a.i./L
96-HOUR NOAEC:	285 mg a.i./L

GROWTH RATE	
72-HOUR EC50:	5661 mg a.i./L
95% CONFIDENCE LIMITS:	5230 and 6067 mg a.i./L
96-HOUR EC10:	1674 mg a.i./L
95% CONFIDENCE LIMITS:	1482 and 1839 mg a.i./L
96-HOUR EC50:	5733 mg a.i./L
95% CONFIDENCE LIMITS:	5659 and 5817 mg a.i./L
96-HOUR EC90:	8849 mg a.i./L
95% CONFIDENCE LIMITS:	8656 and 9081 mg a.i./L
72-HOUR NOAEC:	<285 mg a.i./L
96-HOUR NOAEC:	1077 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 exposure phase of the test was conducted from January 22, 2001 to February 1, 2001. Raw data generated by Wildlife International Ltd and a copy of the final report are filed under Project Number 454A-129 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of the study was to evaluate the toxicity of perfluorobutanesulfonate, potassium salt (PFBS) 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, two "abiotic" replicates (test solution without algae) were prepared at the highest test concentration for analytical sampling. Nominal test concentrations were selected in consultation with the Sponsor and were based upon the results of a range finding test. The nominal test concentrations selected were 313, 625, 1250, 2500, 5000 and 10000 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 for cell density were calculated for each 24 hour interval. EC10, EC50 and EC90 values were calculated, if possible, based upon cell densities, areas under the growth curve and growth rates for

the 72 and 96 hour intervals. The no-observed-adverse-effect-concentration (NOAEC) was determined, when possible, 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 by performing a recovery phase.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "PFBS: 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 June 28, 2000 and was assigned Wildlife International, Ltd. identification number 5292. The test substance was described as a white powder. It was identified as Potassium Perfluoro Butane Sulfonate, AKA . Developmental Product, AKA PFBS, from lot 2. Information provided by the Sponsor indicated a purity of 97.9%. A subsequent revision of the certificate of analysis indicated a purity of 97.3% and an Expiration/Reassessment Date of January 17, 2002. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 313, 625, 1250, 2500, 5000 and 10000 mg a.i./L. A 2-L primary stock solution was prepared in algal medium at a concentration of 10000 mg a.i./L. The primary stock solution was inverted at least 20 times aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with algal medium to prepare 500 mL of the five other test concentrations. All dilutions were inverted to mix. After mixing, 100 mL of test solution was added to the four replicate test chambers for each treatment group. All test solutions appeared clear and colorless. Test concentrations were corrected for the original reported purity of the active ingredient in the test substance (97.9%).

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 the University of Toronto 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 phase, 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 (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 1). The pH of the medium was adjusted to 7.5 ± 0.1 using 10% HCl; 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. The results of analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International, Ltd. are presented in Appendix 2.

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 two mechanical shaker tables 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±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 ± 430 lux. Light intensity was measured at the four corners and the middle of the shaker table daily during the test. 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 group 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:

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

where: A = Area
N_o = 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:

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

where: N_o = Mean measured number of cells/mL at t_o
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:

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

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 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. If the data did not meet the assumptions of normality or homogeneity, the data was transformed to correct the condition. 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 scintillation vials and were analyzed as soon as possible without storage. Analytical procedures used in the analysis of the samples are presented in Appendix 3.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFBS in the test solutions are presented in Table 1 and Appendix 3. Nominal concentrations selected for use in this study were 313, 625, 1250, 2500, 5000 and 10000 mg a.i./L. Samples collected at the beginning of the test had measured concentrations that ranged from 88 to 99% of nominal. Samples collected at 72 hours and test termination had recoveries that ranged from 80 to 92% and 87 to 94% 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 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L.

Measurements

Measurements of temperature and light intensity are presented in Tables 2 and 3, respectively. The temperatures ranged from 23.8 to 24.7°C and were within the range established for the test (24±2°C). The light intensity during the exposure phase of the test ranged from 3930 to 4550 lux and was within the desired range for the test (approximately 3870 to 4730 lux) (Table 3). Measurements of pH ranged from 6.9 go 7.1 on Day 0 and ranged from 7.5 to 8.7 at 96 hours (Table 4). The pH of the "abiotic" replicate at test termination was 7.6.

Data Analyses

The effect of PFBS 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 4, 5 and 6, respectively. EC50 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. EC10 and EC90 values were also calculated for the 72 and 96-hour intervals, where possible.

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 4.5%. After 72 hours of exposure, cell density percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was 32, 33, 44, 62, 83 and 99%, respectively. Dunnett's test showed that cell density was significantly reduced in all treatment groups in comparison to the negative control ($p \leq 0.05$). Consequently, the NOAEC for 72 hour cell density was <285 mg a.i./L, the lowest concentration tested. After 96 hours of exposure, cell density percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was -10, 8.3, 5.6, 46, 76 and 100%, respectively. Dunnett's test showed that cell density was significantly reduced in the 2216, 4561 and 9478 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96 hour cell density was 1077 mg a.i./L.

After 72 hours of exposure, area under the growth curve percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was 26, 31, 42, 60, 80 and 98%, respectively. Dunnett's test showed that area under the growth curve was significantly reduced in all treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 72 hour area under the growth curve was <285 mg a.i./L, the lowest concentration tested. After 96 hours of exposure, area under the growth curve percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was 5.5, 18, 21, 52, 78 and 99%, respectively. Dunnett's test showed that area under the growth curve was significantly reduced in all treatment groups except the 285 mg a.i./L treatment group ($p \leq 0.05$). Consequently, the NOAEC for 96 hour area under the growth curve was 285 mg a.i./L.

After 72 hours of exposure, growth rate percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was 8.2, 8.7, 13, 21, 38 and 92%, respectively. Dunnett's test showed that growth rate was significantly reduced in all treatment goups ($p \leq 0.05$). Consequently, the NOAEC for 72 hour growth rate was <285 mg a.i./L, the lowest concentration tested. After 96 hours of exposure, growth rate percent inhibition in the 285, 563, 1077, 2216, 4561 and 9478 mg a.i./L treatment groups was –1.6, 1.5, 1.0, 10, 24 and 93%, respectively. Dunnett's test showed that growth rate was significantly reduced in the 2216, 4561 and 9478 mg a.i./L treatment groups ($p \leq 0.05$). Consequently, the NOAEC for 96 hour growth rate was 1077 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 PFBS treatment group. However, algal cells in the 2216, 4561 and 9478 mg a.i./L treatment groups appeared enlarged when compared to the negative control.

Reversibility of Growth Inhibition

The 9478 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 six days. Based on the growth observed in the recovery phase, the effect on algal growth was found to 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 1469 mg a.i./L with 95% confidence limits of 81 and 2812 mg a.i./L. The 96-hour EC10, based on area under the growth curve, was 385 mg a.i./L with 95% confidence limits of 191 and 951 mg a.i./L. The 96-hour EC50, based on area under the growth curve, was 2146 mg a.i./L with 95% confidence limits of 1748 and 2706 mg a.i./L. The 96-hour EC90, based on area under the growth curve was 7294 mg a.i./L with 95% confidence limits of 6888 and 7673 mg a.i./L. The 72-hour NOAEC value, based on cell density, area under the growth curve and growth rate, was <285 mg a.i./L, the lowest concentration tested. The 96-hour NOAEC value, based on area under the growth curve, was 285 mg a.i./L. Based on the algal growth observed during the recovery phase, PFBS 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.

Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater 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		
313	0	290	285	91
	72	281		
	96	283		
625	0	567	563	90
	72	563		
	96	558		
1250	0	1146	1077	86
	72	993		
	96	1091		
2500	0	2188	2216	89
	72	2209		
	96	2252		
5000	0	4522	4561	91
	72	4551		
	96	4610		
10000	0	9859	9478	95
	72	9154		
	96	9421		
10000 (Abiotic)	72	9467	9484	95
	96	9501		

[1] Limit of Quantitation (LOQ) was 100 mg a.i./L.

[2] 0-hour samples were collected from individual batches of test solution prepared for the treatment and control group at test initiation.

[3] 72-hour samples were collected from the additional (D) replicate.

[4] 96-hour samples were composites of test solution collected from each of the three replicates per treatment and control group.

Table 2

Temperature Measurements

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater medium

Time (Day)	Temperature (°C)	
	Measurement 1	Measurement 2[1]
0	24.1	24.1
1	24.1	23.8
2	24.3	24.4
3	24.3	24.3
4	24.5	24.5
5 (Recovery)	24.5	24.5
6	24.5	24.5
7	24.5	24.5
8	24.7	24.1
9	24.3	24.4
10	24.4	24.5

[1] With the exception of Day 10, temperature measurement 2 was taken at least 4 hours after measurement 1.

Table 3

Light Intensity Measurements
Shaker Table AQL #6

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater medium

Test Day	Light Intensity Measurements (lux)				
	No. 1	No. 2	No. 3	No. 4	No. 5
0	3960	4010	4490	3990	4370
1	3980	4030	4400	4350	4260
2	3930	4030	4120	4050	4550
3	3960	4110	4380	4100	4280
4	3990	4230	4190	4200	4030
5 (Recovery)	4010	4020	3990	3980	4020
6	3980	4100	4240	4000	4030
7	4970	4990	4130	3980	4160
8	3880	3890	4450	3950	3910
9	3890	3910	3960	4380	3940
10	4110	4060	4410	3890	3970

Table 3 (Continued)

Light Intensity Measurements
Shaker Table AQL #3

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater medium

Test Day	Light Intensity Measurements (lux)				
	No. 1	No. 2	No. 3	No. 4	No. 5
0	4030	3990	4000	4090	4070
1	4000	4610	3890	4040	4410
2	3940	3940	4470	3910	4070
3	3960	4180	4390	4230	3930
4	3980	4060	4410	4180	3890

Table 4

pH Measurements

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater medium

Mean Measured Concentration (mg a.i./L)	pH Measurements	
	0 Hours[1]	96 Hours[2]
Negative Control	6.9	8.7
285	6.9	8.7
563	7.0	8.7
1077	7.0	8.7
2216	7.0	8.7
4561	7.1	8.5
9478	7.1	7.5
9484 (Abiotic)	--	7.6

[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.

Table 5

Mean Cell Densities and Percent Inhibition for Each 24-Hour Interval During the Test[1]

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	31,000	--	179,000	--	1,015,000	--	3,560,000	--
285	30,333	2.2	162,333	9.3	688,333[2]	32	3,920,000	-10
563	27,000	13	138,667	23	673,333[2]	34	3,265,000	8.3
1077	20,667	33	119,000	34	571,667[2]	44	3,360,000	5.6
2216	17,333	44	95,333	47	385,000[2]	62	1,930,000[3]	46
4561	15,333	51	62,333	65	175,333[2]	83	860,000[3]	76
9478	12,000	61	16,000	91	14,667[2]	99	15,333[3]	100

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 Test[1]

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	252,000	--	2,532,000	--	16,620,000	--	71,280,000	--
285	244,000	3.2	2,316,000	8.5	12,284,000[2]	26	67,344,000	5.5
563	204,000	19	1,952,000	23	11,456,000[2]	31	58,476,000[3]	18
1077	128,000	49	1,564,000	38	9,612,000[2]	42	56,552,000[3]	21
2216	88,000	65	1,200,000	53	6,724,000[2]	60	34,264,000[3]	52
4561	64,000	75	756,000	70	3,368,000[2]	80	15,552,000[3]	78
9478	48,000	81	148,000	94	276,000[2]	98	396,000[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 7

Mean Growth Rates and Percent Inhibition for Each 24-Hour Interval During the Test[1]

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	0.0445	--	0.0601	--	0.0640	--	0.0612	--
285	0.0458	-3.0	0.0580	3.4	0.0587[2]	8.2	0.0622	-1.6
563	0.0413	7.3	0.0547	8.9	0.0584[2]	8.7	0.0603	1.5
1077	0.0298	33	0.0515	14	0.0560[2]	13	0.0606	1.0
2216	0.0225	49	0.0469	22	0.0506[2]	21	0.0548[3]	10
4561	0.0173	61	0.0377	37	0.0398[2]	38	0.0464[3]	24
9478	0.0130	71	0.0095	84	0.0052[2]	92	0.0043[3]	93

[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:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater 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	--	4366	<0.0 and 12305	Not Determined	--
48 Hours	Not Determined	--	2631	1644 and 3927	Not Determined	--
72 Hours	<285	--[1]	1469	81 and 2812	6821	5639 and 8187
96 Hours	528	375 and 1895	2347	2018 and 2707	7390	7270 and 7500

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

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:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater 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	--	1,134	<0.0 and 4774	Not Determined	--
48 Hours	Not Determined	--	2,009	24 and 3992	Not Determined	--
72 Hours	<285	--[1]	1,590	328 and 2373	7274	6231 and 8204
96 Hours	385	191 and 951	2,146	1748 and 2706	7294	6888 and 7673

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

Table 10

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

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater 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	--	2195	<0.0 and 8922	Not Determined	--
48 Hours	Not Determined	--	5892	4683 and 7385	Not Determined	--
72 Hours	734	<0.0 and 2207	5661	5230 and 6067	9303	8571 and 9664
96 Hours	1674	1482 and 1839	5733	5659 and 5817	8849	8656 and 9081

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

Table 11

Cell Densities During the Recovery Phase

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater medium

Mean Measured Test Concentration (mg a.i./L)	Cell Densities (Cells/mL[2])		
	Day 0	Day 3	Day 6
Negative Control	18,000	1,785,000	6,140,000
9478[1]	2,000	3,000	980,000

[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.

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

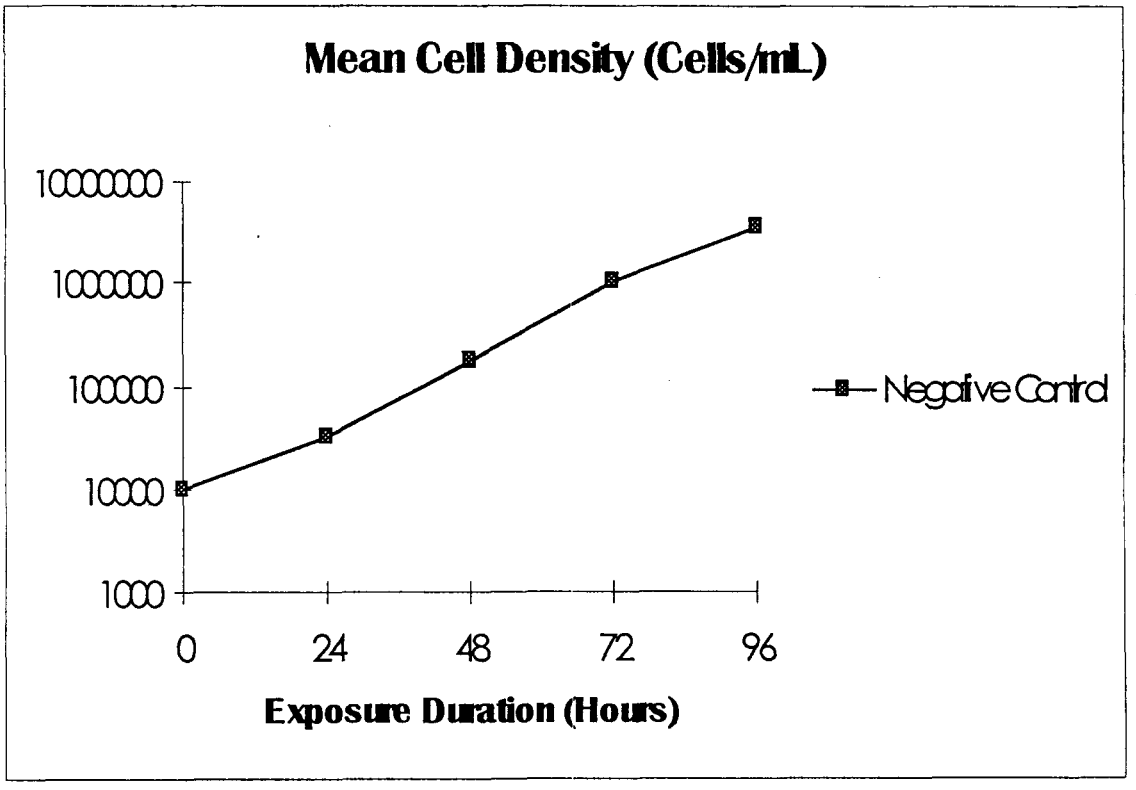


Figure 2. Concentration-Response Curve, Expressed in Cell Density

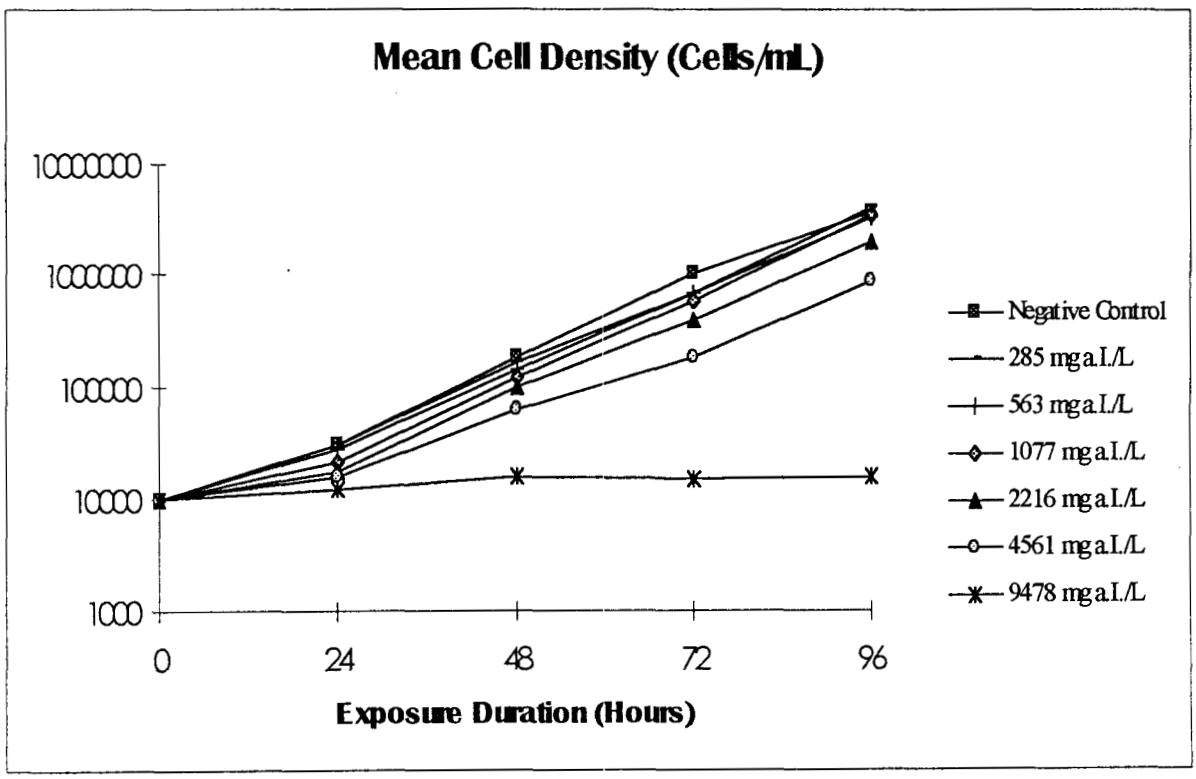
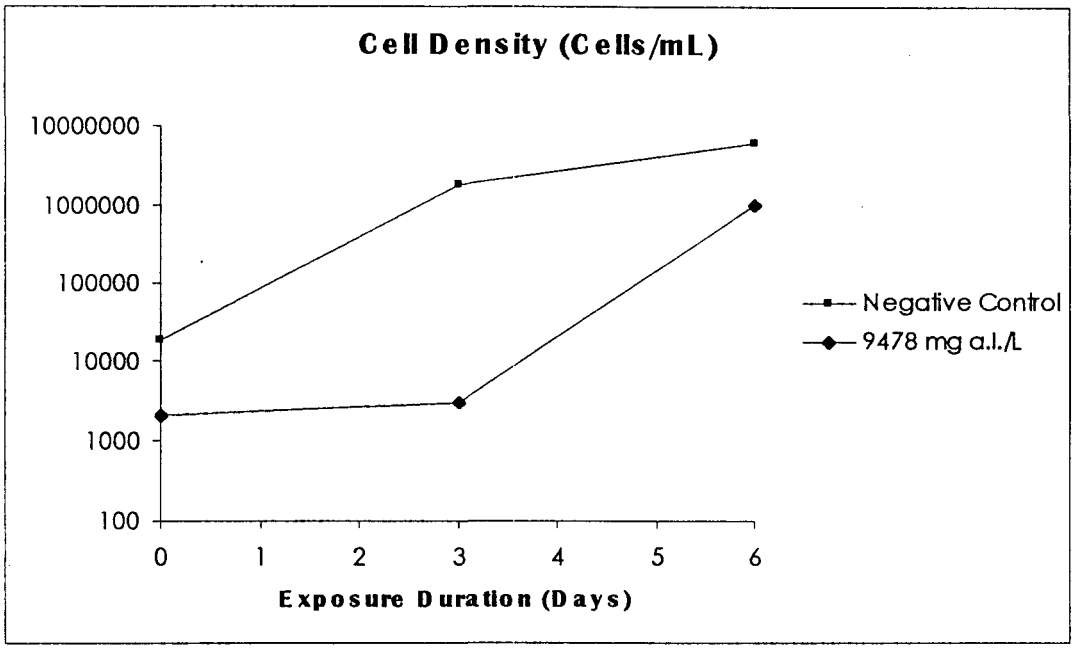


Figure 3. Cell Density During the Recovery Phase



Appendix 1

Freshwater Algal Medium[1]

Sponsor:	3M Corporation
Test Substance:	PFBS
Test Organism:	Freshwater alga, *Selenastrum capricornutum*
Dilution Water:	Freshwater medium

Compound	Nominal Concentration	
$MgCl_2 \bullet 6H_2O$	12.16	mg/L
$CaCl_2 \bullet 2H_2O$	4.41	mg/L
H_3BO_3	0.1855	mg/L
$MnCl_2 \bullet 4H_2O$	0.4154	mg/L
$ZnCl_2$	3.27	µg/L
$FeCl_3 \bullet 6H_2O$	0.1598	mg/L
$CoCl_2 \bullet 6H_2O$	1.428	µg/L
$Na_2MoO_4 \bullet 2H_2O$	7.26	µg/L
$CuCl_2 \bullet 2H_2O$	0.012	µg/L
$Na_2EDTA \bullet 2H_2O$	0.300	mg/L
$NaNO_3$	25.50	mg/L
$MgSO_4 \bullet 7H_2O$	14.70	mg/L
K_2HPO_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 2
Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water[1]

Component	Measured Concentration	Component	Measured Concentration
Pesticides and Organics			
Aclonifen	<0.03 µg/L	Dimethomorf	<0.05 µg/L
Alachlor	<0.01 µg/L	Disulfoton	<0.02 µg/L
Ametryn	<0.01 µg/L	DMST	<0.05 µg/L
Atrazin	<0.01 µg/L	Dodemorf	<0.01 µg/L
Azinphos-ethyl	<0.04 µg/L	Endosulfan-α	<0.01 µg/L
Azinphos-methyl	<0.08 µg/L	Endosulfan-β	<0.01 µg/L
Azoxystrobin	<0.25 µg/L	Endosulfan-sulfaat	<0.02 µg/L
Bifenthrin	<0.05 µg/L	Epoxiconazole	<0.05 µg/L
Bioallethrin	<0.05 µg/L	Eptam	<0.02 µg/L
Bitertanol	<0.05 µg/L	Esfenvaleraat	<0.02 µg/L
Bromacil	<0.05 µg/L	Ethion	<0.05 µg/L
Bromophos	<0.02 µg/L	Ethofumesaat	<0.02 µg/L
Bromophos-ethyl	<0.02 µg/L	Ethoprophos	<0.01 µg/L
Broompropylaat	<0.02 µg/L	Etridiazole	<0.02 µg/L
Bupirimaat	<0.05 µg/L	Etrimphos	<0.05 µg/L
Carbaryl	<0.05 µg/L	Fenarimol	<0.05 µg/L
Carbofuran	<0.03 µg/L	Fenchlorphos	<0.01 µg/L
Carboxin	<0.02 µg/L	Fenitrothion	<0.03 µg/L
Chlorfenvinphos	<0.02 µg/L	Fenoxycarb	<0.03 µg/L
Chloridazon	<0.05 µg/L	Fenpiclonil	<0.05 µg/L
Chlorpropham	<0.02 µg/L	Fenpropathrin	<0.25 µg/L
Chlorpyriphos	<0.01 µg/L	Fenpropimorf	<0.01 µg/L
Chlorpyriphos-methyl	<0.01 µg/L	Fenthion	<0.01 µg/L
Chlorthalonil	<0.04 µg/L	Fenvaleraat	<0.02 µg/L
Coumaphos	<0.02 µg/L	Fluazifop-butyl	<0.02 µg/L
Cyanazin	<0.05 µg/L	Fluoroglycofen-ethyl	<0.02 µg/L
Cyfluthrin	<0.05 µg/L	Fluroxypyr-meptyl	<0.05 µg/L
Cypermethrin	<0.25 µg/L	Flutolanil	<0.02 µg/L
Cyproconazole	<0.05 µg/L	Fonophos	<0.01 µg/L
Deltamethrin	<0.02 µg/L	Furalaxyl	<0.02 µg/L
Demeton	<0.02 µg/L	Heptenophos	<0.02 µg/L
Demeton-o	<0.02 µg/L	Imazalil	<0.01 µg/L
Desethylatrazin	<0.01 µg/L	Iprodion	<0.05 µg/L
Desisopropylatrazin	<0.02 µg/L	Kresoxim-methyl	<0.02 µg/L
Desmetryn	<0.01 µg/L	Lenacil	<0.05 µg/L
Diazinon	<0.01 µg/L	Lindane	<0.02 µg/L
Dichlobenil	<0.01 µg/L	Malathion	<0.02 µg/L
Dichloran	<0.03 µg/L	Metalaxyl	<0.05 µg/L
Dichlorbenzamide	<0.02 µg/L	Metamitron	<0.05 µg/L
Dichlorfenthion	<0.01 µg/L	Metazachlor	<0.02 µg/L
Dichlorfluanid	<0.03 µg/L	Methidathion	<0.02 µg/L

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on October 14 and 15, 1999.

Appendix 2 (Continued)
Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water[1]

Pesticides And Organics (Page 2)			
Component	Measured Concentration	Component	Measured Concentration
Dichlorvos	<0.01 µg/L	Methoxychlor	<0.01 µg/L
Dicofol	<0.25 µg/L	Metolachlor	<0.01 µg/L
Diethyltoluamide	<0.02 µg/L	Metribuzin	<0.02 µg/L
Difenoconazole	<0.03 µg/L	Mevinphos	<0.01 µg/L
Dimethoate	<0.02 µg/L	Nitrothal-Isopropyl	<0.05 µg/L
Paclobutazole	<0.05 µg/L	Pyrifenox-1	<0.01 µg/L
Parathion	<0.01 µg/L	Pyrifenox-2	<0.01 µg/L
Parathion-methyl	<0.01 µg/L	Pyrimethanil	<0.01 µg/L
Penconazole	<0.05 µg/L	Quizalofop-ethyl	<0.02 µg/L
Pendimethalin	<0.03 µg/L	Simazin	<0.01 µg/L
Permethrin-cis	<0.01 µg/L	Sulfotep	<0.02 µg/L
Permethrin-trans	<0.01 µg/L	Tebuconazole	<0.05 µg/L
Phosalon	<0.05 µg/L	Tebufenpyrad	<0.05 µg/L
Phosmet	<0.02 µg/L	Terbutryn	<0.01 µg/L
Phosphamidon-cis	<0.05 µg/L	Terbutylazin	<0.01 µg/L
Pirimicarb	<0.01 µg/L	Tetrachlorvinphos	<0.01 µg/L
Pirimiphos-ethyl	<0.01 µg/L	Tetrahydroftaalimide	<0.05 µg/L
Pirimiphos-methyl	<0.01 µg/L	Tetramethrin	<0.01 µg/L
Prochloraz	<0.02 µg/L	Thiabendazole	<0.05 µg/L
Procymidon	<0.01 µg/L	Thiometon	<0.04 µg/L
Prometryn	<0.01 µg/L	Tolclophos-methyl	<0.01 µg/L
Propachlor	<0.01 µg/L	Tolylfluanid	<0.04 µg/L
Propazin	<0.01 µg/L	Triadimefon	<0.05 µg/L
Propham	<0.02 µg/L	Triadimenol	<0.05 µg/L
Propiconazool	<0.05 µg/L	Triallaat	<0.02 µg/L
Propoxur	<0.03 µg/L	Triazophos	<0.02 µg/L
Propyzamide	<0.02 µg/L	Trifluralin	<0.02 µg/L
Prosulfocarb	<0.02 µg/L	Vamidothion	<0.01 µg/L
Pyrazophos	<0.03 µg/L	Vinchlozolin	<0.01 µg/L
Metals			
Magnesium	11.0 mg/L	Nickel	<1.1 µg/L
Sodium	18.0 mg/L	Copper	<0.7 µg/L
Calcium	29 mg/L	Zinc	<0.25 µg/L
Iron	<0.015 mg/L	Molybdenum	<0.3 µg/L
Potassium	1.1 mg/L	Silver	<0.2 µg/L
Aluminum	<0.02 mg/L	Cadmium	<0.1 µg/L
Manganese	<0.1 µg/L	Arsenic	<0.5 µg/L
Beryllium	<0.2 µg/L	Mercury	<0.025 µg/L
Chromium	<0.5 µg/L	Selenium	<0.5 µg/L
Cobalt	<0.2 µg/L		

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on October 14 and 15, 1999.

Appendix 3

THE ANALYSIS OF PFBS IN FRESHWATER ALGAL MEDIUM

IN SUPPORT OF

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

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFBS: A 96-HOUR TOXICITY TEST with the FRESHWATER ALGA (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-129

3M ENVIRONMENTAL LAB PROJECT NUMBER: E00-1429

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

03-20-01
DATE

MANAGEMENT:

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

3/20/01
DATE

SANITIZED

DEC 0 9 2003

Introduction

Freshwater medium samples were collected from an acute toxicity study designed to determine the effects of PFBS (Perfluoro Butane Sulfonate, Potassium Salt) to the freshwater alga (*Selenastrum capricornutum*). This study was conducted by Wildlife International, Ltd. and identified as Project Number 454A- 129. 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 January 22, 25 and 26, 2001 and were analyzed on each sample receipt day.

Analytical Standard

The analytical standard was received from 3M Environmental Technology and Safety Services on March 27, 2000, assigned Wildlife International, Ltd. Identification number 5216, and stored under ambient conditions. The analytical standard, a white powder, was identified as: Potassium Perfluorobutane Sulfonate expiration date: March 2010. The analytical standard was further identified with the 3M Environmental Laboratory test control and reference number

The test substance had a reported purity of 97.90%. A subsequent revision of the certificate of analysis indicated a purity of 97.3% and an Expiration/Reassessment Date of January 17, 2002. The analytical standard was the same material and lot number as the test substance (Wildlife International, Ltd. Identification number The analytical standard was used to prepare calibration and matrix fortification samples.

Analytical Method

Freshwater medium samples were analyzed according to the method entitled "Analytical Method Validation for the Determination of Perfluorobutane Sulfonate, Potassium Salt (PFBS) in Saltwater and Algal Media" (Wildlife International, Ltd. Project No. 454C-117). Samples were diluted in a 50% methanol : 50% NANOpure® water solution so that they fell within the calibration range of the PFBS methodology. Aliquots of the dilutions were transferred to autosampler vials and submitted for analysis by direct injection. Concentrations of PFBS in freshwater medium samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) interfaced with a Perkin-Elmer API 100LC mass spectrometer (single quadrupole) operated in selective ion monitoring (SIM) detection mode. The mass spectrometer was equipped with a Perkin-Elmer TurboIonSpray ion source. Chromatographic separations were achieved using a Keystone

PRISM RP column (30 mm × 1.5 mm, 3-µm particle size) fitted with a Keystone Javelin C_{18} Guard Cartridge (20 mm × 2 mm). The instrument parameters are summarized in Table 1 and a method flowchart is provided in Figure 1.

Primary and Secondary Stock Solutions.

All primary and secondary stock preparations were adjusted for the purity of the analytical standard (97.90%). A 10.0 mg a.i./mL primary stock solution of PFBS in methanol was prepared by weighing 1.024 g of the analytical standard and bringing to a final volume of 100 mL with methanol. Secondary stock solutions (1000, 100, 10.0, 1.00, and 0.100 mg a.i./L) of PFBS in methanol were prepared by serial volumetric dilution from the primary stock.

Calibration Standards and Calibration Curves

Calibration standards were prepared in 50:50 methanol: NANOpure® water by appropriate dilutions of the 10.0 mg a.i./L stock solution of PFBS in methanol. The calibration standards of PFBS, ranging in concentration from 0.0100 to 0.0500 mg a.i./L, were analyzed with each sample set. Five calibration standards (different concentrations) were analyzed with the samples. The calibration standard series was injected at the beginning and end of each run, and one standard was injected, at a minimum, after every five samples. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFBS in the samples was determined by substituting the peak area responses into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

Limit of Quantitation

The method limit of quantitation (LOQ) for these analyses was set at 100 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (10000).

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 medium was directly fortified (*i.e.* without use of carrier solvent) with PFBS at 150, 2500 and 12000 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 2). Sample concentrations were not corrected for the mean procedural recovery of 93.5%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-129-4, nominal concentration of 1250 mg a.i./L in freshwater medium.

First Initial Volume: 0.100 mL
First Final Volume: 100 mL
Second Initial Volume: 0.250 mL
Second Final Volume: 10.0 mL
Dilution Factor: 40000
PFBS Peak Area: 1385658

Calibration curve equation:
Slope: 43223072
Intercept: 147443.62500
Curve regression weighted 1/x

$$\text{PFBS (mg a.i./L) measured at instrument} = \frac{\text{peak area - (y-intercept)}}{\text{slope}}$$

$$\text{PFBS (mg a.i./L) in sample} = \text{PFBS measured at instrument (mg a.i./L)} \times \text{dilution factor}$$

$$= \frac{1385658 - 147443.62500}{43223072} \times 40000$$

$$= 1146$$

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

$$= \frac{1146}{1250} \times 100 = 91.7\%$$

Calculated with HPLC/MS instrument software: MacQuan, version 1.6.

RESULTS

Sample Analysis

Freshwater medium samples were collected from an acute toxicity study with the freshwater alga (*Selenastrum capricornutum*) at test initiation, January 22, 2001 (Day 0), on January 25, 2001 (Day 3), and at test termination, January 26, 2001 (Day 4). The measured concentrations of PFBS in the samples collected at initiation of exposure of the test organisms (Day 0) ranged from 87.5 to 98.6% of the nominal concentrations. Samples collected at Day 3 had a measured concentration range of 79.5 to 94.7% of nominal values. Samples collected at test termination (Day 4) had a measured concentration range of 87.3% to 95.0% of nominal values (Table 3). Samples from the abiotic 10000 mg a.i./L treatment group were comparable to samples from the 10000 mg a.i./L treatment group with the freshwater alga present (Table 3). 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 operated in Selective Ion Monitoring (SIM) Mode
ION SOURCE:	Perkin-Elmer TurboIonSpray
ANALYTICAL COLUMN:	Keystone PRISM RP (30 mm × 1.5 mm, 3-µm particle size)
GUARD COLUMN:	Keystone Javelin C_{18} cartridge (20 mm × 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	3.00 min
FLOW RATE:	200 µL/min
MOBILE PHASE:	25% NANOpure® Water with 0.1% Ammonium Formate: 75% Methanol
INJECTION VOLUME:	5.0 µL
PFBS PEAK RETENTION TIME:	Approximately 2.2 minutes
PFBS MONITORED MASS:	299.0 amu

Table 2

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-129-)	Sample Type	Concentrations of PFBS (mg a.i./L)		Percent Recovered[1]
		Fortified	Measured[1]	
MAB-1	Matrix Blank	0.00	<LOQ[2]	--
MAB-2	Matrix Blank	0.00	<LOQ	--
MAB-3	Matrix Blank	0.00	<LOQ	--
MAS-1	Matrix Fortification	150	141	93.8
MAS-4	Matrix Fortification	150	138	92.1
MAS-7	Matrix Fortification	150	136	90.9
MAS-2	Matrix Fortification	2500	2338	93.5
MAS-5	Matrix Fortification	2500	2385	95.4
MAS-8	Matrix Fortification	2500	2302	92.1
MAS-3	Matrix Fortification	12000	11460	95.5
MAS-6	Matrix Fortification	12000	11520	96.0
MAS-9	Matrix Fortification	12000	11050	92.1
				Mean = 93.5
				Standard Deviation = 1.82
				CV =1.95%
				N = 9

[1] Measured and Percent Recovered values were calculated using MacQuan, version 1.6 software. Manual calculations may vary slightly.

[2] The limit of quantitation (LOQ) was 100 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (10000).

Table 3

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

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-129-)	Sampling Time (Day)	PFBS Measured Concentration[1] (mg a.i./L)	Percent of Nominal[1]
0.0	1	0	< LOQ[2]	--
	8	3	< LOQ	--
	16	4	< LOQ	--
313	2	0	290	92.6
	9	3	281	89.9
	17	4	283	90.3
625	3	0	567	90.7
	10	3	563	90.2
	18	4	558	89.3
1250	4	0	1146	91.7
	11	3	993	79.5
	19	4	1091	87.3
2500	5	0	2188	87.5
	12	3	2209	88.3
	20	4	2252	90.1
5000	6	0	4522	90.5
	13	3	4551	91.0
	21	4	4610	92.2
10000	7	0	9859	98.6
	14	3	9154	91.5
	22	4	9421	94.2
10000 (Abiotic)	15	3	9467	94.7
	23	4	9501	95.0

[1] Measured and Percent of Nominal values were calculated using MacQuan, version 1.6 software. Manual calculations may vary slightly.

[2] The limit of quantitation (LOQ) was 100 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (10000).

METHOD OUTLINE FOR THE ANALYSIS OF PFBS IN FRESHWATER MEDIUM

Prepare each matrix fortification sample by weighing the requisite amount of PFBS test substance on an analytical balance and transferring directly into a Class A volumetric flask partially filled with freshwater medium. Rinse weighing paper and the sides of the flask with repeat freshwater medium rinses. Swirl the flask to dissolve the test substance and then bring to final volume with freshwater medium. Sonicate, as appropriate, and mix with several repeat inversions. The matrix blank is unfortified freshwater medium.

↓

Centrifuge samples, as appropriate, at approximately 2500 rpm for approximately 15 minutes.

↓

Prepare appropriate dilutions of study and QC samples to within the calibration range of the PFBS methodology: Partially fill Class A volumetric flasks with 50% methanol : 50% NANOpure® water dilution solvent. Add the appropriate volume of sample and bring to volume with dilution solvent. Perform secondary dilutions as necessary. Process matrix blank samples 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 PFBS in freshwater medium.

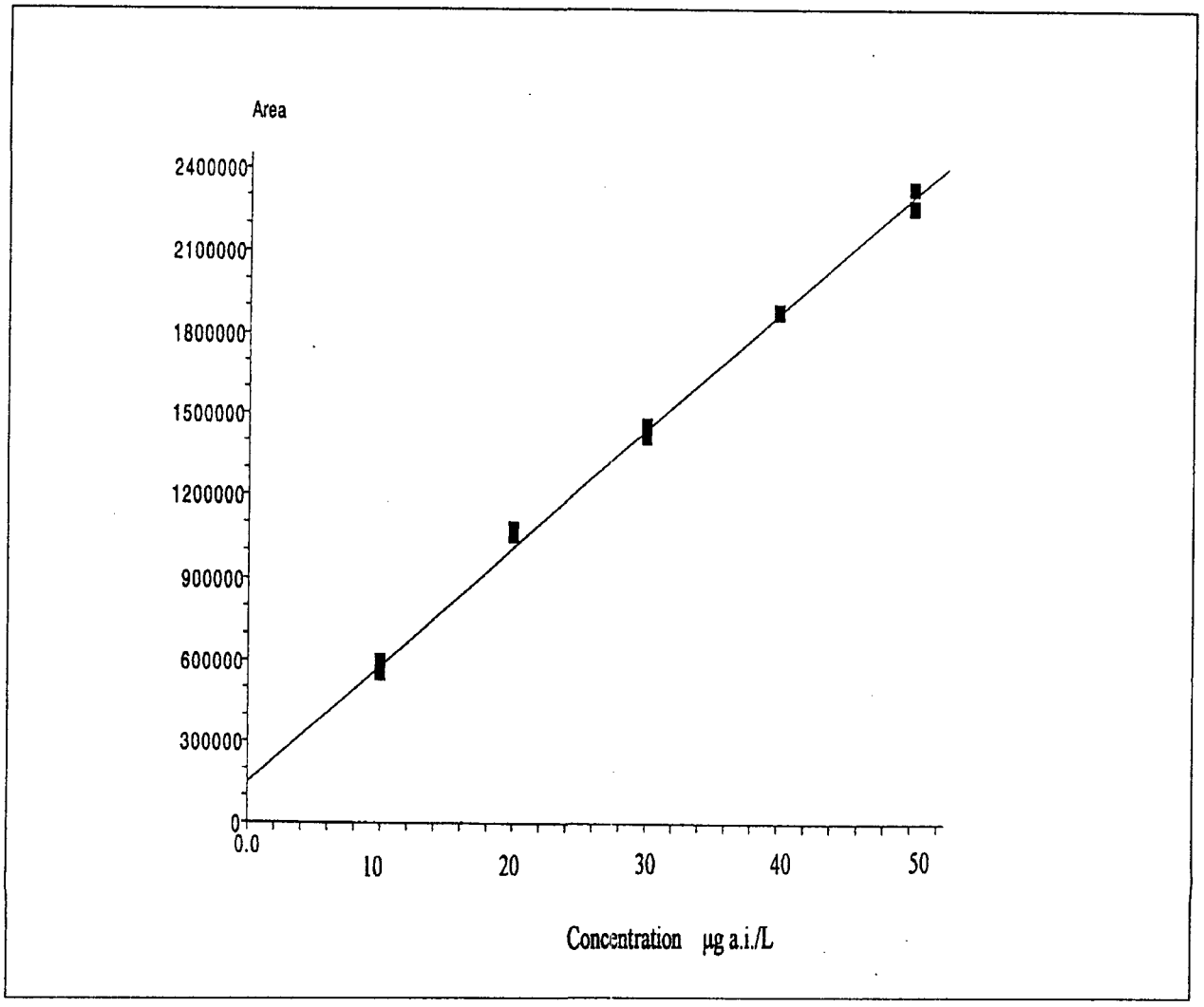


Figure 2. A typical calibration curve for PFBS. Slope = 43223072; Intercept = 147443.62500; r = 0.9986.

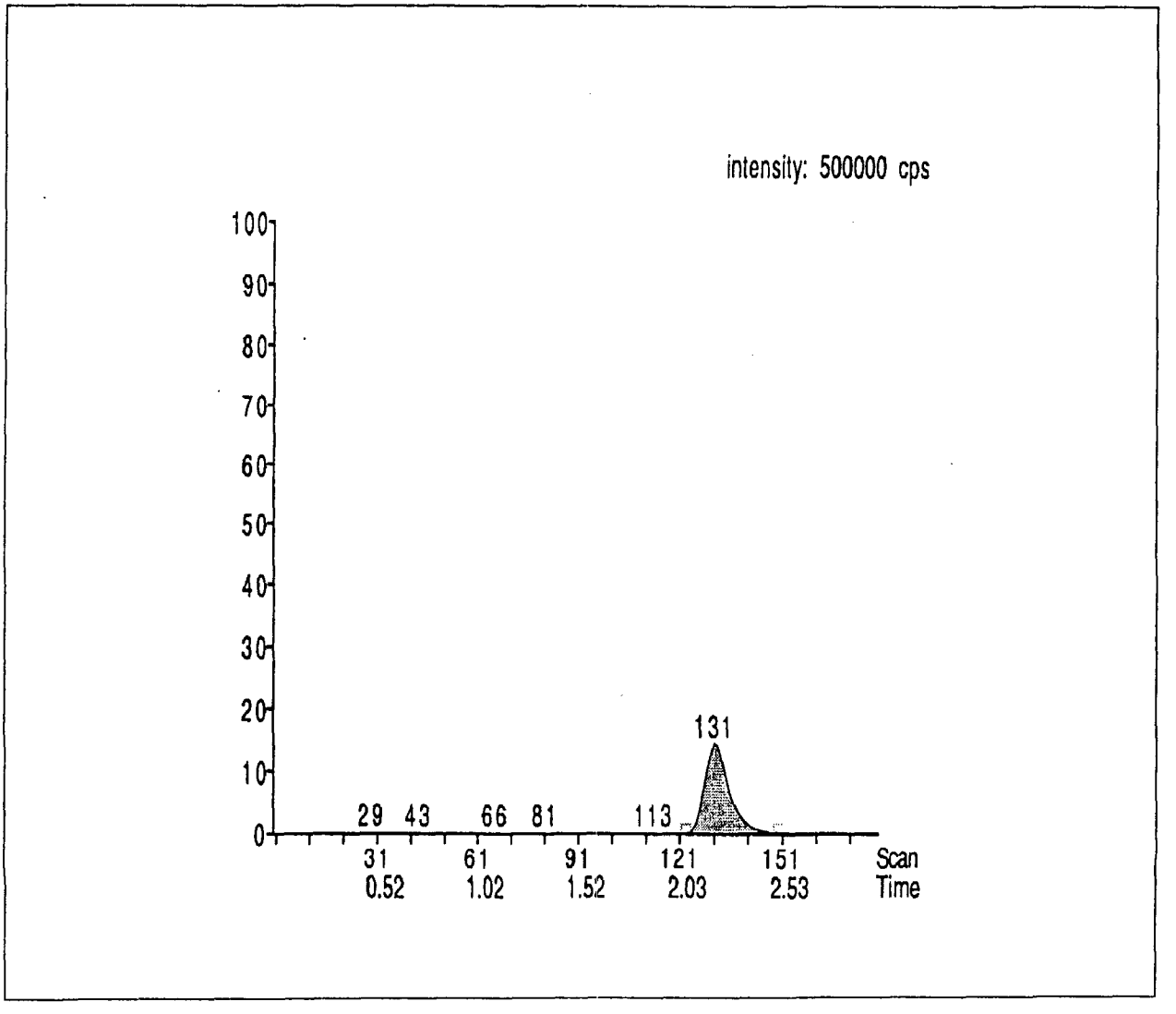


Figure 3. A representative ion chromatogram of a low-level (0.0100 mg a.i./L) PFBS standard.

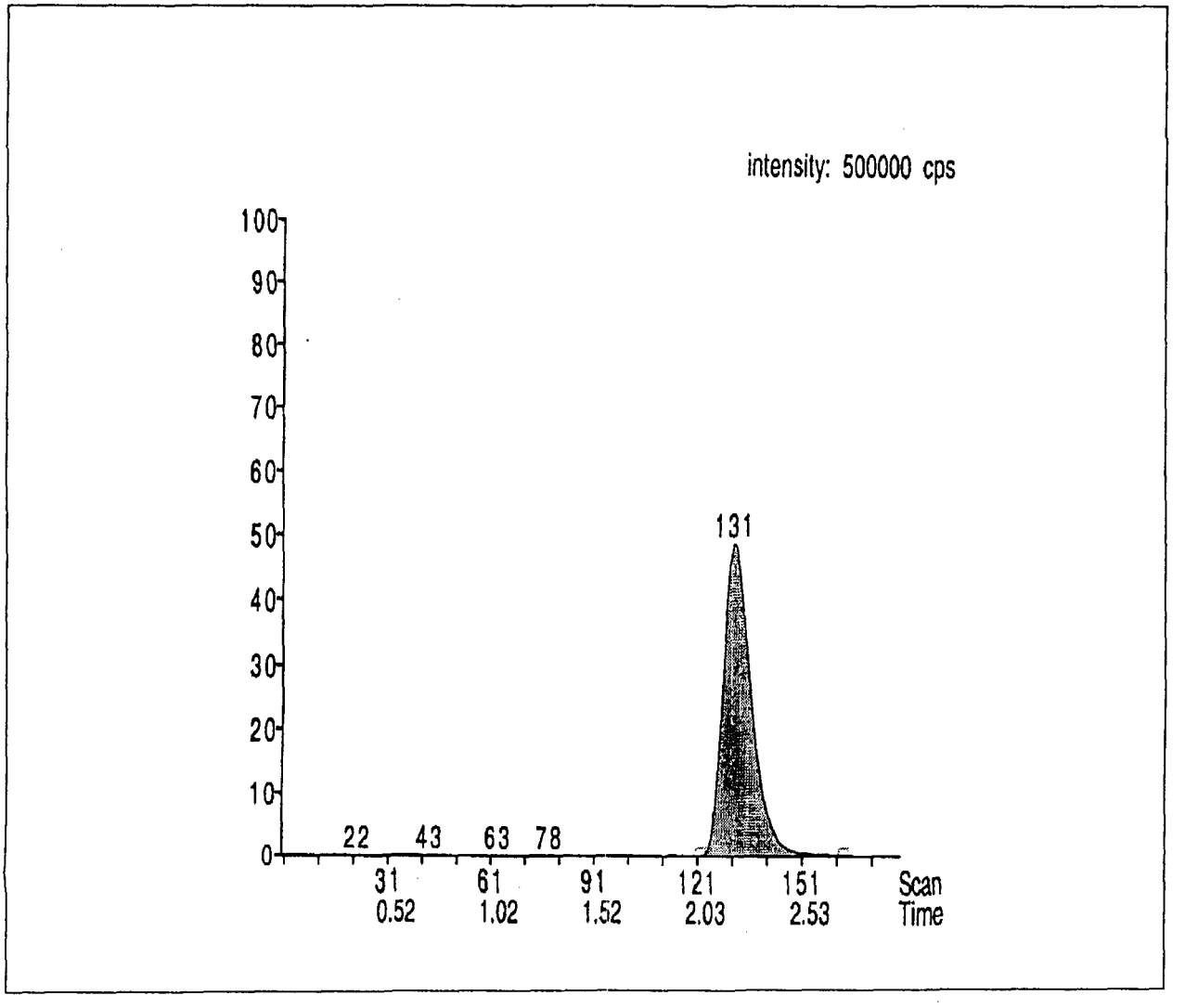


Figure 4. A representative ion chromatogram of a high-level (0.0500 mg a.i./L) PFBS standard.

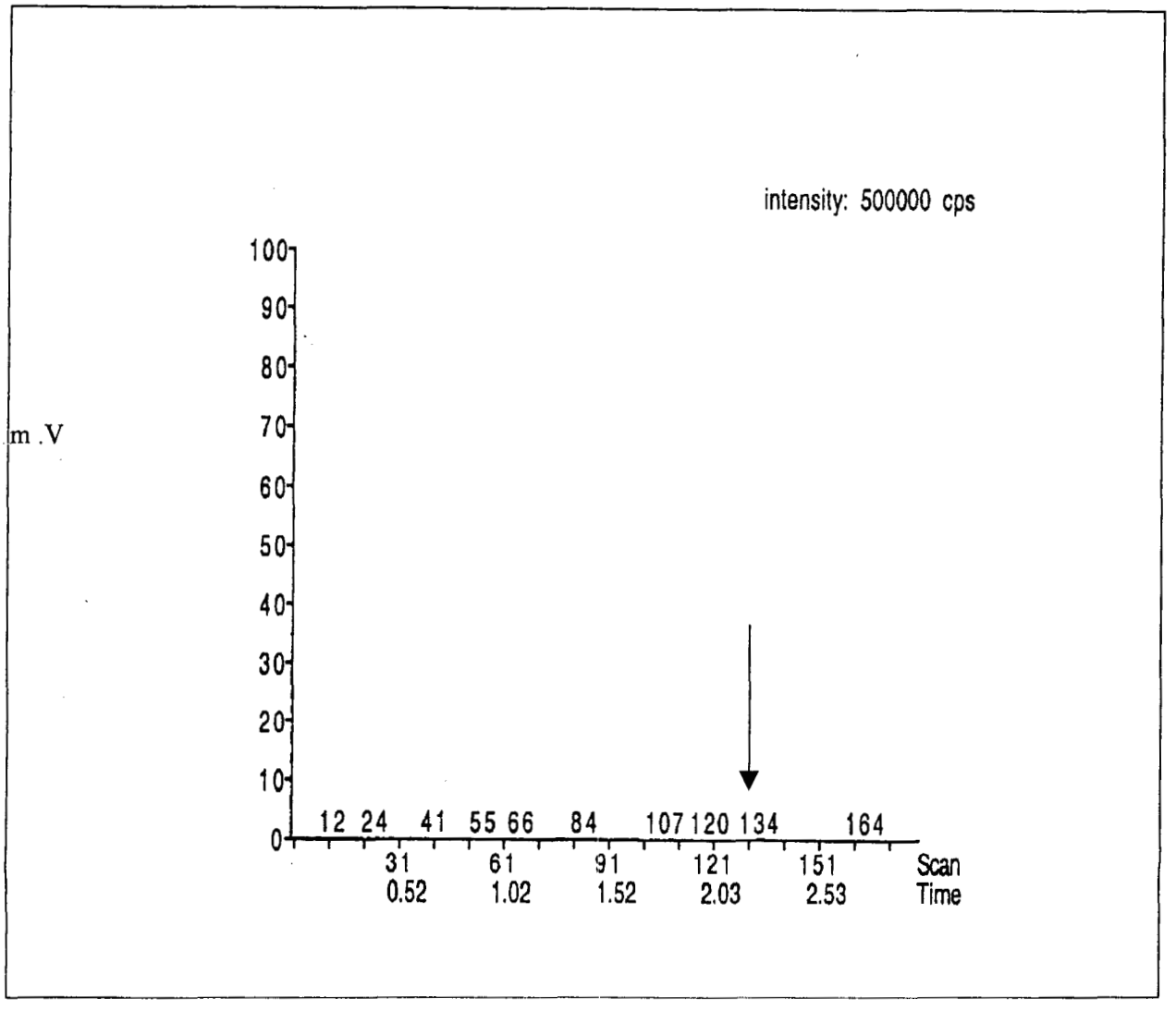


Figure 5. A representative ion chromatogram of a matrix blank sample (454A-129-MAB-1). The arrow indicates the retention time of PFBS.

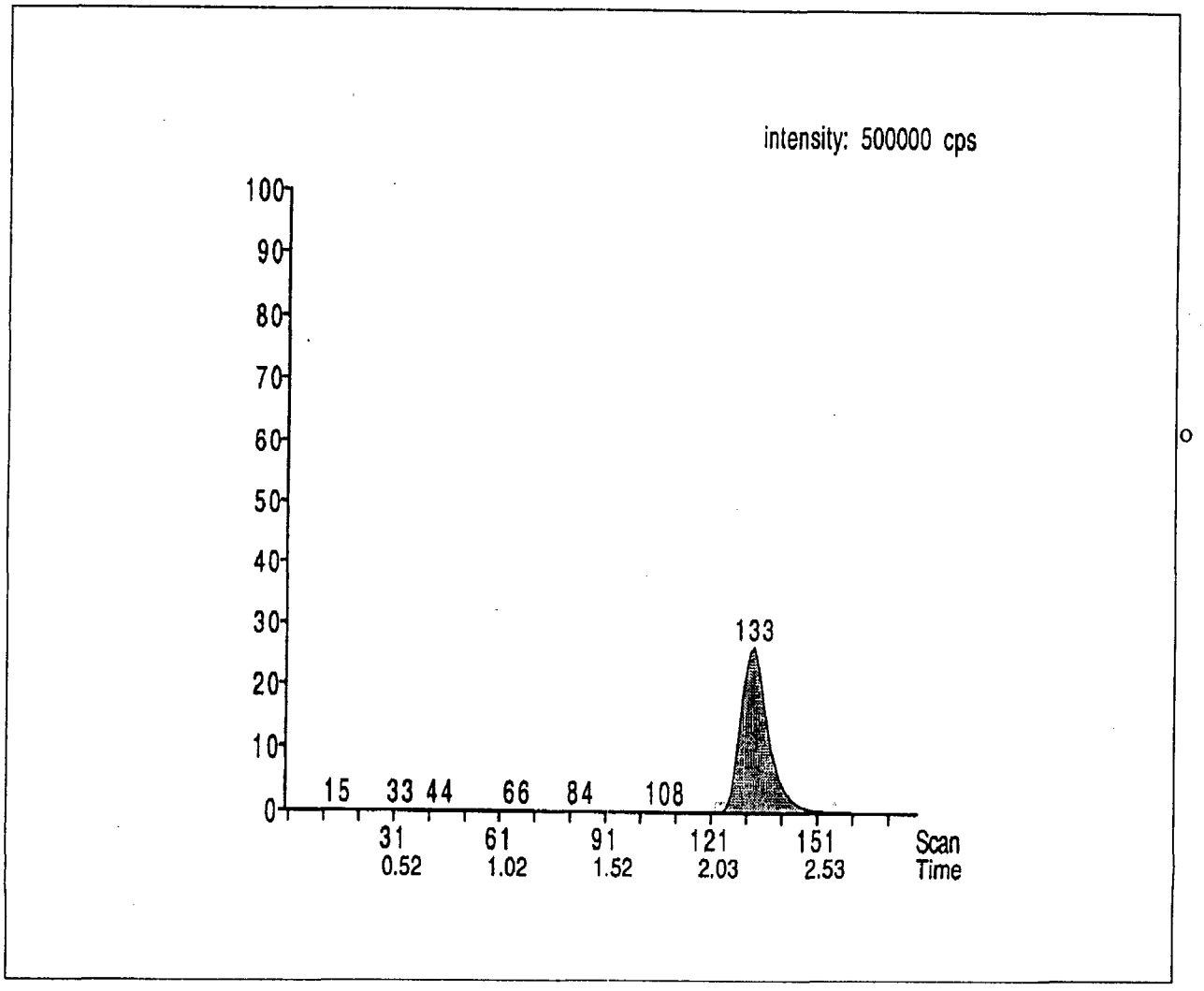


Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-129-MAS-2, nominal PFBS concentration of 2500 mg a.i./L, dilution factor = 100000x).

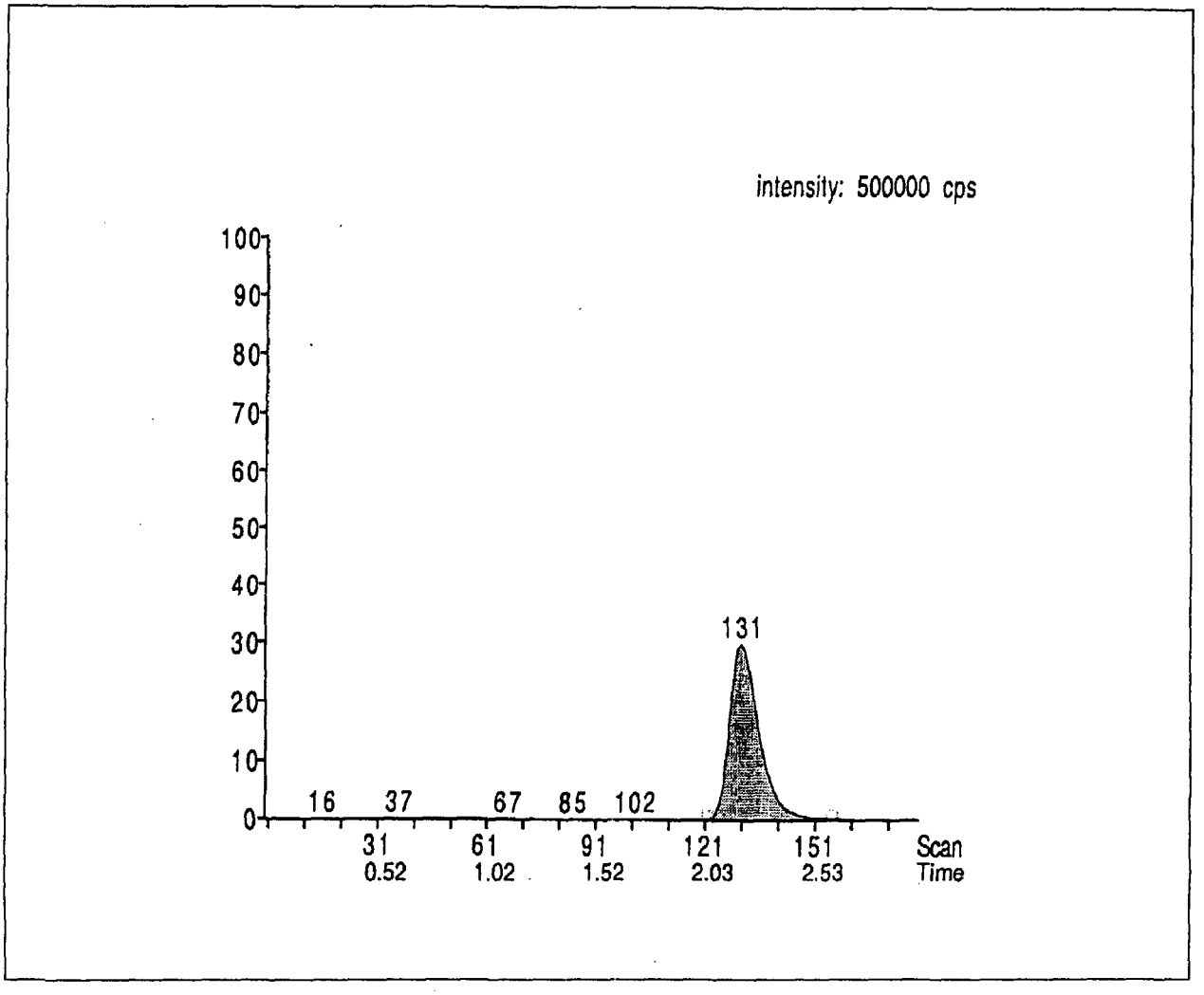


Figure 7. A representative ion chromatogram of a test sample (454A-129-4, nominal PFBS concentration of 1250 mg a.i./L, dilution factor = 40000x).

Appendix 4

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

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	21,000	168,000	1,200,000	3,735,000
	B	25,000	184,000	1,045,000	3,420,000
	C	47,000	185,000	800,000	3,525,000
285	A	29,000	157,000	625,000	4,005,000
	B	26,000	152,000	730,000	3,930,000
	C	36,000	178,000	710,000	3,825,000
563	A	25,000	136,000	645,000	3,120,000
	B	30,000	126,000	625,000	3,195,000
	C	26,000	154,000	750,000	3,480,000
1077	A	24,000	133,000	720,000	3,750,000
	B	17,000	106,000	465,000	3,180,000
	C	21,000	118,000	530,000	3,150,000
2216	A	19,000	88,000	430,000	2,025,000
	B	14,000	97,000	330,000	1,845,000
	C	19,000	101,000	395,000	1,920,000
4561	A	12,000	51,000	171,000	850,000
	B	17,000	80,000	188,000	870,000
	C	17,000	56,000	167,000	860,000
9478	A	17,000	13,000	16,000	19,000
	B	4,000	15,000	16,000	15,000
	C	15,000	20,000	12,000	12,000

[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 5

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

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	132,000	2,160,000	18,336,000	77,316,000
	B	180,000	2,448,000	16,956,000	70,296,000
	C	444,000	2,988,000	14,568,000	66,228,000
285	A	228,000	2,220,000	11,364,000	66,684,000
	B	192,000	2,088,000	12,432,000	68,112,000
	C	312,000	2,640,000	13,056,000	67,236,000
563	A	180,000	1,872,000	11,004,000	55,944,000
	B	240,000	1,872,000	10,644,000	56,244,000
	C	192,000	2,112,000	12,720,000	63,240,000
1077	A	168,000	1,812,000	11,808,000	65,208,000
	B	84,000	1,320,000	7,932,000	51,432,000
	C	132,000	1,560,000	9,096,000	53,016,000
2216	A	108,000	1,152,000	7,128,000	36,348,000
	B	48,000	1,140,000	6,024,000	31,884,000
	C	108,000	1,308,000	7,020,000	34,560,000
4561	A	24,000	540,000	2,964,000	14,976,000
	B	84,000	1,008,000	3,984,000	16,440,000
	C	84,000	720,000	3,156,000	15,240,000
9478	A	84,000	204,000	312,000	492,000
	B	0	0	132,000	264,000
	C	60,000	240,000	384,000	432,000

Appendix 6

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

Sponsor: 3M Corporation
Test Substance: PFBS
Test Organism: Freshwater alga, *Selenastrum capricornutum*
Dilution Water: Freshwater 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	0.0309	0.0588	0.0665	0.0617
	B	0.0382	0.0607	0.0646	0.0608
	C	0.0645	0.0608	0.0609	0.0611
285	A	0.0444	0.0574	0.0574	0.0624
	B	0.0398	0.0567	0.0596	0.0622
	C	0.0534	0.0600	0.0592	0.0619
563	A	0.0382	0.0544	0.0579	0.0598
	B	0.0458	0.0528	0.0574	0.0601
	C	0.0398	0.0570	0.0600	0.0610
1077	A	0.0365	0.0539	0.0594	0.0617
	B	0.0221	0.0492	0.0533	0.0600
	C	0.0309	0.0514	0.0551	0.0599
2216	A	0.0267	0.0453	0.0522	0.0553
	B	0.0140	0.0473	0.0486	0.0544
	C	0.0267	0.0482	0.0511	0.0548
4561	A	0.0076	0.0339	0.0394	0.0463
	B	0.0221	0.0433	0.0407	0.0465
	C	0.0221	0.0359	0.0391	0.0464
9478	A	0.0221	0.0055	0.0065	0.0067
	B	0.000	0.0084	0.0065	0.0042
	C	0.0169	0.0144	0.0025	0.0019

Appendix 7

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 and test concentrations.
2. The protocol was amended to provide the correct freshwater algal medium constituents.
3. Two light intensity measurements during the recovery phase were outside of the acceptable range.

Appendix 8

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. VanHoven, Ph.D., Scientist
4. Kurt R. Drottar, Senior Biologist
5. Cary A. Sutherland, Laboratory Supervisor
6. Debbie Desjardins, Biologist