

Protocol. PFOS: A 7-Day Toxicity Test with Duckweed  
(*Lemna gibba* G3)

## PROTOCOL

### PFOS: A 7-DAY TOXICITY TEST WITH DUCKWEED (*Lemna gibba* G3)

U.S. Environmental Protection Agency  
Series 850 - Ecological Effects Test Guidelines  
OPPTS Number 850.4400

3M Lab Request No. U2723

Submitted to

3M Corporation  
Environmental Laboratory  
935 Bush Avenue  
St. Paul, Minnesota 55106

***Wildlife International, Ltd.***

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

September 17, 1999

PFOS: A 7-DAY TOXICITY TEST  
WITH DUCKWEED (*Lemna gibba* G3)

SPONSOR:

3M Corporation  
Environmental Laboratory  
935 Bush Avenue  
St. Paul, Minnesota 55106

SPONSOR'S REPRESENTATIVE:

Rochelle R. Robideau

TESTING FACILITY:

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

STUDY DIRECTOR:

~~Kurt Drott~~ Cary Sutherland  
Senior Aquatic Biologist

HK 1/10/00

LABORATORY MANAGEMENT:

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

FOR LABORATORY USE ONLY

|                                                                          |                                         |
|--------------------------------------------------------------------------|-----------------------------------------|
| Proposed Dates:                                                          |                                         |
| Experimental<br>Start Date: _____                                        | Experimental<br>Termination Date: _____ |
| Project No.: <u>454A-111</u>                                             |                                         |
| Test Concentrations: _____                                               |                                         |
| Test Substance No.: _____ Reference Substance No. (if applicable): _____ |                                         |

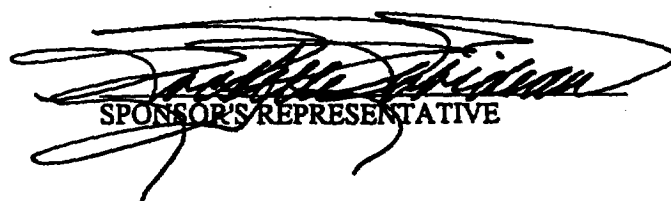
PROTOCOL APPROVAL

  
STUDY DIRECTOR

1/28/00  
DATE

  
LABORATORY MANAGEMENT

1/28/00  
DATE

  
SPONSOR'S REPRESENTATIVE

1/16/00  
DATE

### INTRODUCTION

Wildlife International, Ltd. will conduct a seven-day toxicity test with the duckweed, *Lemna gibba* G3, 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 850.4400 (1). 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 Perfluorooctane Sulfonic Acid, Potassium Salt (hereafter referred to as PFOS) to duckweed, *Lemna gibba* G3, under static test conditions.

### EXPERIMENTAL DESIGN

Fronds of duckweed, *Lemna gibba* G3, will be exposed to a geometric series of at least six test concentrations and a negative (culture medium) control for seven days. Nominal test concentrations will be chosen in consultation with the Sponsor based upon information such as rangefinding toxicity data, known toxicity information or physical/chemical properties of PFOS. Target concentrations will not exceed 100 mg/L or the solubility limit of the test substance in water. Generally, each concentration of PFOS 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.

Uniform, healthy looking plants will be removed from the stock culture for testing. Five plants totalling approximately 15 fronds will be added to each test chamber. Care will be taken to ensure that the number of fronds in each test chamber is approximately equal. Three "biological" replicates per treatment will be tested. One or more additional "analytical" replicates will be included in the test, as needed, to provide test solution for concentration verification on Days 3, 5 and 7 of the exposure. An abiotic replicate at the highest concentration will be included in the test and will be sampled on Days 3, 5 and 7. In order to control bias, the position of the test chambers 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 test results will be calculated based on direct counts of the numbers of duckweed fronds. These measurements will be taken on Day 3, Day 5 and at the end of the test. Observations of chlorosis, break-up of duckweed colonies, changes in color, root destruction and other abnormal appearances will also be documented. An IC10, IC50 and IC90 values (e.g., the toxicant concentration that produces a 10%, 50%

or 90% reduction in frond number relative to the controls) will be calculated based on total frond numbers at Days 3, 5 and 7 of the exposure period. The no observed adverse effect concentration (NOAEC), which represents the highest concentration that induces no adverse effects on plant and frond production or appearance, will be determined based upon evaluation of the statistical results, the dose-response pattern, and other observed effects.

### 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.

#### Test Solution Preparation

The desired concentrations of PFOS will be obtained by preparing a single stock solution in medium and diluting the appropriate volume of stock solution with medium. The test solutions may also be prepared using secondary dilutions of the primary stock, based upon the concentration desired.

The test substance will be administered to the test organism in culture medium. This route of administration was selected because it represents the most likely route of exposure to duckweed, which exist suspended on a water surface with roots extending into the water column.

#### Test Organism

The test species will be the duckweed, *Lemna gibba* G3. This species is representative of an important group of organisms and was selected for use in the test based upon past use and ease of handling in the laboratory. Original duckweed plants will be obtained from the United States Department of Agriculture, Washington, D.C. or another suitable supplier. Stock cultures will be maintained in culture

medium at Wildlife International, Ltd. Duckweed used in toxicity tests will be obtained from stock cultures that have been actively growing in this medium for at least 2 weeks.

#### Culture Medium

Culture medium prepared according to Wildlife International, Ltd. Standard Operating Procedures will be used as dilution water. The concentrations of the medium components are presented in Table 1. Stock nutrient solutions will be prepared by adding reagent-grade or better 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 (0.22 $\mu$ m) or autoclaved prior to use in the test. The pH of the medium should be  $5.0 \pm 0.1$ . 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 most recent GLP compliant 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 250-mL Nalgene® beakers covered with disposable petri dishes and containing 100 mL of test solution. The test chambers will be indiscriminately positioned on a shelf in an environmental chamber. Test chambers will be labeled with the project number, test concentration, and replicate.

#### Environmental Conditions

The test will be conducted at  $25 \pm 2^\circ\text{C}$  under continuous warm-white fluorescent lighting at an intensity of  $5000 \pm 750$  lux. Light intensity will be measured at five locations surrounding the test chambers on Day 0 of the test. Temperature will be measured twice daily in a container of water adjacent to the test chambers in the environmental chamber using a liquid-in-glass mercury thermometer.

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 for pH measurements at test initiation will be collected from the individual batches of test solution prepared for each treatment and control group. At test termination, pH will be measured in pooled samples of test solution collected from each of the three replicates of each respective treatment and control group.

Biological Measurements

Growth, defined as an increase in the total number of fronds, will be assessed on Day 3, Day 5 and at the end of the test. Additionally, the total number of duckweed plants present in each test chamber will be determined at test termination. The numbers of chlorotic, necrotic and dead fronds will be enumerated. Chlorotic fronds are those possessing areas of bleached color progressing from green to yellow. Necrotic fronds are those with localized regions of dead or decaying tissue, usually surrounded by healthy tissue. Fronds with no apparent living tissue, usually all brown, black, white, yellow or clear, will be considered dead. The duckweed plants will be observed for the presence of root destruction (i.e., dead or shortened roots in a treatment group compared to the controls) and breakup of duckweed colonies (i.e., an increased number of free-floating individual fronds in a treatment group compared to the controls). Any other abnormalities in frond or plant appearance will also be documented.

Sampling for Analytical Measurements

Samples of the exposure solutions will be collected on Days 0, 3, 5 and 7 and analyzed for test substance concentrations. The termination day samples may be filtered (0.45  $\mu$ m), as necessary, to remove plant debris prior to analysis. Day 3 and 5 samples will be collected from the extra replicate test chamber(s) included in the test to provide samples for that analysis and from the abiotic replicates. Samples will be placed in an appropriate storage container (e.g., polyethylene, or polypropylene bottle) and will be analyzed immediately, or stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

| Experimental Group                     | Day 0 | Day 3          | Day 5          | Day 7          |
|----------------------------------------|-------|----------------|----------------|----------------|
| Control                                | 1     | 1              | 1              | 1              |
| Solvent Control (if needed)            | 1     | 1              | 1              | 1              |
| Level 1-Low Concentration              | 1     | 1              | 1              | 1              |
| Level 2                                | 1     | 1              | 1              | 1              |
| Level 3                                | 1     | 1              | 1              | 1              |
| Level 4                                | 1     | 1              | 1              | 1              |
| Level 5                                | 1     | 1              | 1              | 1              |
| Level 6-High Concentration             | 1     | 2 <sup>1</sup> | 2 <sup>1</sup> | 2 <sup>1</sup> |
| Totals                                 | 8     | 9              | 9              | 9              |
| <sup>1</sup> Includes abiotic samples. |       |                |                |                |

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.

## 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. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

## Statistical Analyses

Percent inhibition will be calculated for each treatment and control group using the following formula:

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

The Day 7 IC50 value will be determined, when possible, using linear interpolation or other suitable techniques (3) with treatment response (frond number) and exposure concentration data. The percentages of dead, chlorotic and necrotic fronds will be calculated for each treatment and control group as the number of dead, chlorotic or necrotic fronds to the total number of fronds present in each test chamber.

The data will be evaluated for normality and homogeneity of variances. If these assumptions are met, Dunnett's procedure or the Bonferroni t-test (4) may be used to evaluate the data. If the assumptions of normality and homogeneity of variances are not met, non-parametric statistical methods such as the Kruskal-Wallis test (4) may be used to evaluate the data. A no observed adverse effect concentration (NOAEC) will be determined based upon evaluation of the statistical results, the dose-response pattern and other observed effects (e.g., percent chlorosis, necrosis, etc.).

## 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 plants.
5. Culture conditions.
6. Growth measurements.
7. Calculation and preparation of test concentrations.
8. Observations.
9. If applicable, 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 on which the study was initiated and completed. It is the responsibility of the Sponsor to provide the final date that data are recorded for chemistry pathology and/or supporting evaluations that may be generated at other laboratories.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
5. The test substance identified by name, chemical abstracts number or code number, strength, purity, and composition or other appropriate characteristics, if provided by the Sponsor.
6. Stability and the solubility of the test substances 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 system, including the source of the test organisms and their scientific name.
9. A description of the preparation of the test solutions, methods used to allocate organisms to the test chambers and begin the test, numbers of organisms and chambers per treatment, and duration of the test.
10. A description of all circumstances that may have affected the quality or integrity of the data.
11. The name of the Study Director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.

12. A description of the transformations, calculations, or 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 these analyses.
13. Statistical methods employed for analyzing the data.
14. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
15. The location where all raw data and the final report are to be stored.
16. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
17. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes shall be made in the form of an amendment issued by the Study Director. The amendment shall clearly identify the part of the final report that is being amended and the reasons for the alteration. Amendments shall be signed and dated by the Study Director.

#### CHANGING OF PROTOCOL

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

#### GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

**REFERENCES**

- 1 U.S. Environmental Protection Agency. 1996. Series 850 - Ecological Effects Test Guidelines (draft), OPPTS Number 850.4400: *Aquatic Plant Toxicity Test Using Lemna spp., Tiers I and II.*
- 2 ASTM Standard Guide 1415-91 E. 1991. *Standard Guide for Conducting Static Toxicity Tests with Lemna gibba* G3.
- 3 Norberg-King, T.J. 1993. *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach (Version 2.0).* U.S. Environmental Protection Agency. Environmental Research Laboratory. Duluth, Minnesota.
- 4 West, Inc. and D. D. Gulley. 1996. *TOXSTAT Release 3.5.* Western EcoSystems Technology, Inc. Cheyenne, Wyoming.

TABLE 1

## M-HOAGLAND'S MEDIUM WITHOUT EDTA OR SUCROSE CONSTITUENTS

| Compound                                             | Nominal Concentration |      |
|------------------------------------------------------|-----------------------|------|
| H <sub>3</sub> BO <sub>3</sub>                       | 2.86                  | mg/L |
| ZnSO <sub>4</sub> •7H <sub>2</sub> O                 | 0.22                  | mg/L |
| Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O  | 0.12                  | mg/L |
| CuSO <sub>4</sub> •5H <sub>2</sub> O                 | 0.08                  | mg/L |
| MnCl <sub>2</sub> •4H <sub>2</sub> O                 | 3.62                  | mg/L |
| KH <sub>2</sub> PO <sub>4</sub>                      | 0.68                  | g/L  |
| KNO <sub>3</sub>                                     | 1.515                 | g/L  |
| Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O | 1.180                 | g/L  |
| MgSO <sub>4</sub> •7H <sub>2</sub> O                 | 0.492                 | g/L  |
| FeCl <sub>3</sub> •6H <sub>2</sub> O                 | 5.4                   | mg/L |
| Tartaric Acid                                        | 3.0                   | mg/L |

The pH of the medium will be adjusted, as necessary, to  $5.0 \pm 0.1$  using 0.1 N NaOH or 10% HCL.

## APPENDIX I

## IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

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

Internal Standard: 1,1,2,2H,H,H,H Perfluorooctane Sulfonic AcidTest Substance Sample Code or Batch Number: Lot 217Test Substance Purity (% Active Ingredient): 98.9 Expiration Date: 2008

## II. Test Substance Characterization

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

## III. Test Substance Storage Conditions

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

Ambient

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

Other pertinent stability information:

## IV. Test Concentrations:

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

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

## V. Toxicity Information:

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

Aquatic:

Invertebrate Toxicity (EC/LC50)

Fish Toxicity (LC50)

Daphnia magna: 27 mg/LRainbow Trout: 11 mg/LDaphnia magna: 50 mg/LFathead Minnow: 38 mg/L

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

Please see MSDS

APPENDIX II

Analytical Method Provided by Sponsor

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

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

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

## WILDLIFE INTERNATIONAL LTD.

## AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 7-DAY TOXICITY TEST WITH DUCKWEED (*Lemna gibba* G3)

PROTOCOL NO: 454/091799/LEM7D/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-111

EFFECTIVE DATE: February 11, 2000

3M LAB REQUEST NO.: U2723

AMENDMENT: Culture Medium, Page 5Change: The pH of the medium should be  $5.0 \pm 0.1$ .Add: The pH of the medium should be  $7.5 \pm 0.1$ .

REASON: To change test medium from Hoagland's to 20X AAP medium.

AMENDMENT: TABLE 1, Page 11

Remove: Entire table.

Add:

TABLE 1

## 20X AAP MEDIUM

| Component                                           | Nominal Concentration |      |
|-----------------------------------------------------|-----------------------|------|
| MgCl <sub>2</sub> •6H <sub>2</sub> O                | 243.2                 | mg/L |
| CaCl <sub>2</sub> •2H <sub>2</sub> O                | 88.0                  | mg/L |
| H <sub>3</sub> BO <sub>3</sub>                      | 3.712                 | mg/L |
| MnCl <sub>2</sub> •4H <sub>2</sub> O                | 8.32                  | mg/L |
| ZnCl <sub>2</sub>                                   | 65.6                  | µg/L |
| FeCl <sub>3</sub> •6H <sub>2</sub> O                | 3.196                 | mg/L |
| CoCl <sub>2</sub> •6H <sub>2</sub> O                | 28.56                 | µg/L |
| Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O | 145.2                 | µg/L |
| CuCl <sub>2</sub> •2H <sub>2</sub> O                | 0.240                 | µg/L |
| Na <sub>2</sub> EDTA•2H <sub>2</sub> O              | 6.00                  | mg/L |
| NaNO <sub>3</sub>                                   | 510.0                 | mg/L |
| MgSO <sub>4</sub> •7H <sub>2</sub> O                | 294.0                 | mg/L |
| K <sub>2</sub> HPO <sub>4</sub>                     | 20.88                 | mg/L |
| NaHCO <sub>3</sub>                                  | 300.0                 | mg/L |

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

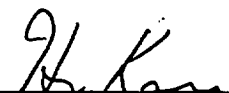
OK  
3/1/2000

WILDLIFE INTERNATIONAL LTD.

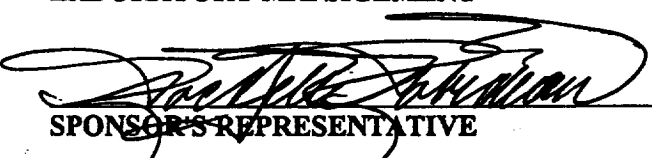
REASON: To change test medium from Hoagland's to 20X AAP medium.

  
STUDY DIRECTOR

3/2/00  
DATE

  
LABORATORY MANAGEMENT

3/2/00  
DATE

  
SPONSOR'S REPRESENTATIVE

3/3/00  
DATE



WILDLIFE INTERNATIONAL LTD.

## AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 7-DAY TOXICITY TEST WITH DUCKWEED (*Lemna gibba* G3)

PROTOCOL NO: 454/091799/LEM7D/SUB454

AMENDMENT NO.: 2

SPONSOR: 3M Corporation

PROJECT NO.: 454A-111

EFFECTIVE DATE: February 22, 2000

3M LAB REQUEST NO.: U2723

AMENDMENT: Page 2

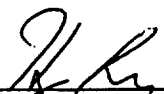
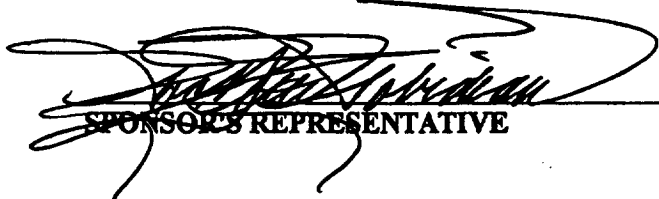
Add: Experimental Start Date: March 3, 2000

Experimental Termination Date: March 10, 2000

Test Concentrations: Negative Control, 13, 25, 50, 100, 200 and 400 mg a.i./L

Test Substance No.: 4675

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

  
STUDY DIRECTOR3/2/00  
DATE  
LABORATORY MANAGEMENT3/2/00  
DATE  
SPONSOR'S REPRESENTATIVE3/3/00  
DATE  
3/1/00

## WILDLIFE INTERNATIONAL LTD.

## AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR TOXICITY TEST WITH THE MARINE DIATOM (*Skeletonema costatum*)

PROTOCOL NO: 454/091799/SKE96/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-113

EFFECTIVE DATE: February 28, 2000

3M LAB REQUEST NO.: U2723

AMENDMENT: Page 2

Add: Experimental Start Date: March 3, 2000  
Experimental Termination Date: March 7, 2000  
Test Concentrations: Negative Control and 4.0 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: Experimental Design Page 3

Change: The marine diatom, *Skeletonema costatum*, will be exposed to a geometric series of six test concentrations and a negative (culture medium) control for 96 hours.

To: The marine diatom, *Skeletonema costatum*, will be exposed to a test concentration at the limit of solubility and a negative (culture medium) control for 96 hours.

REASON: To change experimental design to a limit test.

AMENDMENT: Experimental Design Page 3

Change: Test solutions will be inoculated with approximately 77,000 cells/mL. Test solutions will be inoculated with 10,000 cells/mL.

To: Test solutions will be inoculated with approximately 77,000 cells/mL.

REASON: Typographical error.

AMENDMENT: Experimental Design Page 3

Change: Three "biological" replicates per experimental group will be prepared for evaluating cell densities.

To: Six "biological" replicates per experimental group will be prepared for evaluating cell densities.

REASON: To change experimental design to a limit test.

Reviewed by QA (SC) 3-1-00

WILDLIFE INTERNATIONAL LTD.**AMENDMENT: Sampling for Analytical Measurements, Page 7**

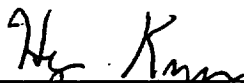
**Change:** Day 4 samples will be composited solutions from the three replicates remaining at the end of the test.

**To:** Day 4 samples will be composited solutions from the six replicates remaining at the end of the test.

**REASON:** To change analytical sampling based on additional replicates provided in the limit test.

  
\_\_\_\_\_  
**STUDY DIRECTOR**

3/2/00  
\_\_\_\_\_  
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