PFOS: An Activated Sludge, Respiration Inhibition Test

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

TEST SUBSTANCE

Identity: Perfluorooctanesulfonate; may also be referred to as PFOS or FC-95. (1-Octanesulfonic acid, 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-, potassium salt, CAS # 2795-39-3)

Remarks: The test substance is a white powder. Sample was taken from 3M lot number 217. Sample was stored under ambient conditions prior to testing. Purity determined to be 90.49% by LC/MS, ^{1}H-HMR, ^{19}F-NMR and elemental analyses techniques.

METHOD

Method: OECD 209
Test type: Static acute
GLP: Yes
Year Completed: Study completed 1999. Report completed 2000
Analytical monitoring: Dissolved oxygen concentrations.
Statistical methods: Probit analysis using the computer software of C.E. Stephan.
Test organism source: Activated sludge collected from the Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland.

Test conditions

Dilution water: NANOpure®
Synthetic Sewage:

1 liter municipal water
16.0 g peptone
11.0 g meat extract
3.0 g urea
0.7 g NaCl
0.4 g $CaCl_2$ $2H_2O$
0.2 g $MgSO_4$ $7H_2O$
2.8 g K_2HPO_4

Reference and test solution preparation: A stock solution of the reference substance, 3,5-dichlorophenol, was prepared by dissolving 500 mg in 10 mL of 1N NaOH, diluted to 30 mL with NANOpure® water, then brought to the point of incipient precipitation with 1N H_2SO_4, and diluted to 1 L with NANOpure® water. The pH of the reference solution was measured to be 7.18.

PFOS was added directly to test vessels rather than volumetric addition of a stock solution. This method was deemed appropriate based on the observed solubility of the test substance in water.
Test vessels: Mixtures were prepared and aerated in 500 mL Erlenmeyer flasks and then transferred into 300 mL Biochemical Oxygen Demand (BOD) bottles
Number of concentrations: 7 plus 3 reference controls and 2 Blank controls
Temperature: 19-21°C
Total Suspended Solids and pH for sludge on day of testing: 4380 mg/L and 7.87 respectively.
Element Basis: Respiration inhibition as determined by dissolved oxygen concentration.

Method Remarks: Stock solutions of PFOS that were prepared at nominal concentration of approximately 500 and 1000 mg/L in NANOpure® water contained test material that was not in solution after 20-minutes of sonication. Therefore, direct weight addition was employed to administer PFOS to the test system.

Test mixtures were prepared at 15-minute intervals and aerated until the contact time of the test substance with the activated sludge was three hours. After 3-hours of contact time, dissolved oxygen was measured over a period of up to 10-minutes.

RESULTS

Nominal concentrations: Two blank controls, three reference substance controls, 0.90, 2.7, 9.0, 27, 90, 271, 905 mg/L test material solutions

Statistical Analyses: EC_{50} values were calculated for the reference material by probit analysis using the computer software of C.E. Stephan. An EC_{50} value could not be calculated for the test substance.

Analytical Methodology: Analysis of DO concentrations in all test solutions were performed at Wildlife International Ltd. using a YSI Model 50B Dissolved Oxygen Meter. Dissolved oxygen readings were recorded every 10 seconds for 10 minutes or until the dissolved oxygen dropped below 1.0 mg/L

Respiration Rates and Percent Inhibitions

Treatment	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	39.6	NA
Control 2	41.1	NA
3,5-dichlorophenol 3 mg/L	31.1	22.9
3,5-dichlorophenol 15 mg/L	14.6	63.8
3,5-dichlorophenol 50 mg/L	5.1	87.4
Test substance 0.90 mg/L	38.9	3.6
Test substance 2.7 mg/L	35.1	13.0
Test substance 9.0 mg/L	33.5	17.0
Test substance 27 mg/L	37.9	6.1
Test substance 90 mg/L	32.7	19.0
Test substance 271 mg/L	28.1	30.4
Test substance 905 mg/L	24.7	38.8

Control response: satisfactory

CONCLUSIONS

The test substance exhibited a maximum inhibitory effect of 38% upon respiration at a nominal test substance concentration of 905 mg/L. The EC_{50} (respiration inhibition) is therefore greater than the solubility of the test substance.

Submitter: 3M Company, Environmental Laboratory, P.O. Box 33331, St. Paul, Minnesota, 55133

DATA QUALITY

Reliability: Klimisch ranking 1.

REFERENCES

This study was conducted at Wildlife International, Ltd. Easton, MD at the request of the 3M Company.

OTHER

Last changed: 5/3/00

PFOS:
AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454E-101

3M LAB REQUEST NUMBER: U2723

AUTHORS:

Edward C. Schaefer
R. Scott Flaggs

STUDY INITIATION DATE: December 11, 1998

STUDY COMPLETION DATE: March 23, 1999

AMENDED REPORT DATE: April 28, 2000

SUBMITTED TO:

3M
Environmental Laboratory
Bldg 2-3E-09
St. Paul, Minnesota 55133

Wildlife International, Ltd.

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

AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: An Activated Sludge, Respiration Inhibition Test

3M LAB REQUEST NUMBER: U2723

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454E-101

STUDY COMPLETION: March 23, 1999

REPORT AMENDED: April 28, 2000

This study was conducted in compliance with the U.S. Environmental Protection Agency in 40 CFR Part 160; 17 August 1989; OECD Principles of Good Laboratory Practices [OCDE/GD(92)32] Environment Monograph No. 45; Paris 1992; and Japan MAFF 59 NohSan, Notification No, 3850, Agricultural Production Bureau, with the following exceptions:

The reference substance, obtained from Aldrich Chemical Company (Milwaukee, WI), was not characterized in compliance with Good Laboratory Practice Standards. A certificate of analysis has been supplied by Aldrich.

Stability, homogeneity and verification of the test and reference substances in the mixtures were not determined.

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:

Edward C. Schaefer
Manager, Biodegradation

4/28/2000
DATE

AMENDED

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with the U.S. Environmental Protection Agency in 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practices [OCDE/GD(92)32] Environment Monograph No. 45 [OCDE/GD(92)32] and Japan MAFF, 59 NohSan, Notification No, 3850, Agricultural Production Bureau; Wildlife International, Ltd. Standard Operating Procedures and the study protocol. 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 Chamber Preparation	December 22, 1998	December 22, 1998	December 23, 1998
DO Measurements	December 22, 1998	December 22, 1999	December 22, 1998
Raw Data and Draft Report	January 27 and 28, 1999	January 28, 1999	February 3, 1999
Final Report	March 23, 1999	March 23, 1999	March 23, 1999
Amended Final Report	April 28, 2000	April 28, 2000	April 28, 2000

Marshall T. Hynson
Quality Assurance Program Supervisor

DATE 4/28/2000

AMENDED

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: An Activated Sludge, Respiration Inhibition Test

3M LAB REQUEST NUMBER: U2723

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454E-101

STUDY DIRECTOR:

Edward C. Schaefer, B.S. — DATE 4/25/2000
Manager, Biodegradation

MANAGEMENT:

Henry O. Krueger, Ph.D. — DATE 4/28/00
Director, Aquatic Toxicology and Non-Target Plants

SPONSOR'S REPRESENTATIVE:

Susan A. Beach — DATE 5/1/00
3M Corporation

AMENDED

STUDY INFORMATION

Study Initiation Date:	December 11, 1998
Experimental Start Date:	December 22, 1998
Experimental Termination Date:	December 22, 1998
Study Completion Date:	March 23, 1999
Amended Final Report Date:	April 28, 2000

Study Director:	Edward C. Schaefer B.S.
Sponsor:	3M Environmental Laboratory St. Paul, Minnesota 55133
Sponsor's Representative:	Susan A. Beach
Study Personnel:	Edward C. Schaefer, B.S., Manager, Biodegradation Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants Abul Siddiqui, B.S., Scientist, Biodegradation Sheri Trumbull, Technologist, Biodegradation R. Scott Flaggs, B.S., Biologist, Biodegradation

TABLE OF CONTENTS

TABLE OF CONTENTS

- Continued -

TABLE

FIGURES

APPENDICES

AMENDED

ABSTRACT

The effect of the test substance on activated sludge microorganisms was assessed by the Activated Sludge Respiration Inhibition Test Method (OECD Guideline 209). The test contained a control, reference and treatment groups. The control group was used to determine the background respiration rate of the sludge and was not dosed with the test or reference substance. The reference group was dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at concentrations of 3, 15 and 50 mg/L. The test substance was dosed at 0.90, 2.7, 9.0, 27, 90, 271 and 905 mg a.i./L. After an exposure period of 3 hours, the respiration rates of the test solutions were measured using a dissolved oxygen meter. The respiration rates in the two controls were 39.8 and 41.1 mg O_2/L/hr, or a difference of 3.2%. The difference in control respiration rates was within the 15% difference limit established for the test. The validity of the test was further supported by the results from the 3,5-dichlorophenol reference group, which resulted in an EC50 of 9.0 mg/L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test.

Differences in O_2 respiration rates between the control group and test substance treatments were observed at all test substance concentrations. Following is a summary of the results.

Treatment	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	39.6	NA
Control 2	41.1	NA
3,5-dichlorophenol 3 mg/L	31.3	22.9
3,5-dichlorophenol 15 mg/L	14.6	63.8
3,5-dichlorophenol 50 mg/L	5.1	87.4
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 0.90 mg a.i./L	38.9	3.6
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 2.7 mg a.i./L	35.1	13.0
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 9.0 mg a.i./L	33.5	17.0
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 27 mg a.i./L	37.9	6.1
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 90 mg a.i./L	32.7	19.0
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 271 mg a.i./L	28.1	30.4
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) 905 mg a.i./L	24.7	38.8

INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. biodegradation facility in Easton, Maryland. Original raw data generated by Wildlife International Ltd. and a copy of the original final report are filed under Project Number 454E-101 in the archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to assess the effects of the test substance on activated sludge microorganisms by measuring the respiration rate.

EXPERIMENTAL DESIGN

The test contained control, reference, and treatment groups. The control group was used to determine the background respiration rate of the sludge and was not exposed to the test or reference substances. The reference group was dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at concentrations of 3, 15, and 50 mg/L. The test substance was tested at 0.90, 2.7, 9.0, 27, 90, 271 and 905 mg a.i./L.

MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, "PFOS: An Activated Sludge, Respiration Inhibition Test," (Appendix IV). The protocol was based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1) and Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2).

Test Substance

The test substance, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), was received at Wildlife International Ltd. on October 29, 1998 and was assigned Wildlife International Ltd. identification number 4675.

The test substance was stored under ambient conditions. Following is a description of the test substance used in this study:

Name:	Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)
Lot Number:	217
Physical Description:	White powder
Expiration Date:	2008
Purity:	90.49%
Water Solubility:	Slight
Storage Conditions:	Ambient Room temperature

On the day of administration, the test substance was added to the appropriate test flasks via direct weight addition. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated March 9, 2000 indicated a purity of 90.49%.

Reference Substance

A stock solution of the reference substance, 3,5-dichlorophenol was prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with NANOpure® water. While stirring, enough 1N H_2SO_4 was added to reach the point of incipient precipitation. The solution of 3,5-dichlorophenol then was diluted to 1 L with NANOpure® water. The pH of an aliquot of the reference substance stock solution was measured (pH = 7.18). Following is a description of reference substance used in this study.

Name:	3,5-dichlorophenol
Manufacturer:	Aldrich Chemical Co., Milwaukee, WI
Lot Number:	00410KZ
Physical Description:	White crystal
Handling Precautions:	Standard laboratory precautions
Expiration Date:	March 13, 2000
Purity:	97%
Water Solubility:	7.39 g/L
Storage Conditions:	Ambient
CAS Number:	591-35-5

Route of Exposure and Duration

The reference substance was administered by volumetric addition. Volumetric addition is the most appropriate route of administration of a substance in an aqueous solution. The test substance was administered by direct weight addition. Direct weight addition was selected as the most appropriate route of administration based on the observed solubility of the test substance in water. Stock solutions that were prepared at nominal concentrations of 1g/L and 0.5 g/L in NANOpure® water and sonicated for approximately 20 minutes contained test material that was not in solution. The duration of the exposure was 3 hours.

Test System

The biological test system was a consortium of microorganisms common to the activated sludge treatment process. The organisms responsible for the decomposition of organic materials are principally aerobic, and facultative bacteria. The test system was chosen because it is representative of a treatment process that may receive the test substance.

Test Conditions and Apparatus

Control, reference, and treatment test mixtures were incubated at 20 ± 2°C and aerated for 3 hours at a rate sufficient to provide aerobic conditions and maintain solids in suspension. The mixtures were prepared and aerated in 500 mL Erlenmeyer flasks and then transferred into 300 mL Biochemical Oxygen Demand (BOD) bottles to conduct the dissolved oxygen (DO) measurements. Test mixtures were identified by project number, test substance ID, test concentration and bottle number, when appropriate.

Test Inoculum

Activated sludge was collected from the Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland on December 21, 1998. The Prospect Bay facility receives wastes from predominately domestic sources. The sludge was sieved using a 2 mm screen and then settled for approximately 30 minutes. The supernatant above the settled solids was drained and the total suspended solids (TSS) concentration of the settled sludge was determined. Based on the result, the concentration of the sludge was adjusted to 4000 mg/L (± 10%) by diluting with municipal water.

Approximately 50 mL of synthetic sewage (Protocol, Appendix IV) was added to each liter of activated sludge and the sludge was aerated overnight at 20 ± 2°C. Before use, the pH and total suspended solids concentration of the activated sludge were determined.

Procedure

Test mixtures were prepared at 15 minute intervals starting with the first control. The control contained 9.6 mL of synthetic sewage, 120 mL of inoculum, and enough municipal water to bring the total volume up to 300 mL. The mixture was promptly aerated at a rate sufficient to provide aerobic conditions and keep the solids in suspension. Subsequent mixtures contained 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate amount of test substance or volume of reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control was prepared. All mixtures were aerated for three hours.

Sample Analysis

After three hours of aeration, the contents of the first vessel were transferred to a BOD bottle and the respiration rate was measured over a period of up to 10 minutes. Dissolved oxygen readings were recorded every 10 seconds for 10 minutes or until the DO dropped below 1.0 mg/L, whichever came first using a YSI Model 50B Dissolved Oxygen Meter. The respiration rate in subsequent vessels was determined in an identical manner at 15 minute intervals so that the contact time of the test substance with the activated sludge was three hours.

Calculations

A respiration rate was calculated for each test mixture and expressed in mg O_2/L/hour. The rate was calculated using DO values between approximately 6.5 mg O_2/L and 2.5 mg O_2/L, or over a 10 minute period if the DO did not reach approximately 2.5 mg O_2/L. The respiration rate was calculated using the following equation:

Respiration rate (mg O_2/L/hour) = Oxygen uptake over the calculation period/duration of calculation period

The percent inhibition for each test substance concentration was calculated using the following equation and plotted against the logarithm of test substance concentration (Appendix III):

$$Percent\ Inhibition = 1 - \frac{2R_s}{RC_1 + RC_2} \times 100$$

where:

R_s	=	oxygen consumption rate at a given concentration of the test substance
RC_1	=	oxygen consumption rate, Control 1
RC_2	=	oxygen consumption rate, Control 2

Statistical Analyses

When the dose response pattern allows for the calculation of an EC50 value, the data are analyzed using the computer program of C.E. Stephan (3). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average, or binomial probability with nonlinear interpolation (4, 5, 6). In this study, probit analysis was used to evaluate inhibition in the reference group. An EC50 value could not be calculated for the test substance.

RESULTS AND DISCUSSION

The pH of the reference stock solution was 7.18. Respiration rates and percent inhibitions are presented in Table 1. Dissolved oxygen readings recorded during the test are presented in tabular and graphical forms in Appendices I and II, respectively. The temperature range recorded the day of the test was 19-21°C and was within the limits specified in the test guideline. The measured total suspended solids (TSS) concentration and pH of the sludge on the day of testing was 4380 mg/L and 7.87, respectively.

The respiration rates in the two controls were 39.6 and 41.1 mg O_2/L/hr, or a difference of 3.7%. The validity of the test was further supported by the results from the 3,5-dichlorophenol reference group, which resulted in an EC50 of 9.0 mg/L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test. The 95% confidence limits for the EC50 were 6.9 and 11.4 mg/L.

The inhibitory effects upon respiration at test substance concentrations analyzed (0.90, 2.7, 9.0, 27, 90, 271 and 905 mg a.i./L) ranged from 3.6% to 38.8%. Respiration rates of the test substance concentrations evaluated ranged from 24.7 mg O_2/L/hr (at 905 mg a.i./L) to 38.9 mg O_2/L/hr (at 1 mg/L).

CONCLUSION

The maximum inhibitory effect upon respiration from Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) was observed at a test substance concentration of 905 mg a.i./L, but only reached 38.8%. The EC50 is therefore greater than 905 mg a.i./L.

REFERENCES

1. **Organisation for Economic Cooperation and Development.** 1989. *Activated Sludge Respiration Inhibition Test.* OECD Guideline 209.

2. **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.11, *Activated Sludge Respiration Inhibition Test.*

3. **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

4. **Finney, D.J.** 1971. *Statistical Methods in Biological Assay*, second edition. Griffin Press, London.

5. **Thompson, W.R.** 1947. *Bacteriological Reviews*, Vol. II, No. 2: 115-145.

6. **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

Table 1

Respiration Rates and Percent Inhibitions

Treatment		Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1		39.6	NA
Control 2		41.1	NA
3,5-dichlorophenol	3 mg/L	31.1	22.9%
3,5-dichlorophenol	15 mg/L	14.6	63.8%
3,5-dichlorophenol	50 mg/L	5.1	87.4%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	0.90 mg a.i./L	38.9	3.6%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	2.7 mg a.i./L	35.1	13.0%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	9.0 mg a.i./L	33.5	17.0%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	27 mg a.i./L	37.9	6.1%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	90 mg a.i./L	32.7	19.0%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	271 mg a.i./L	28.1	30.4%
Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)	905 mg a.i./L	24.7	38.8%

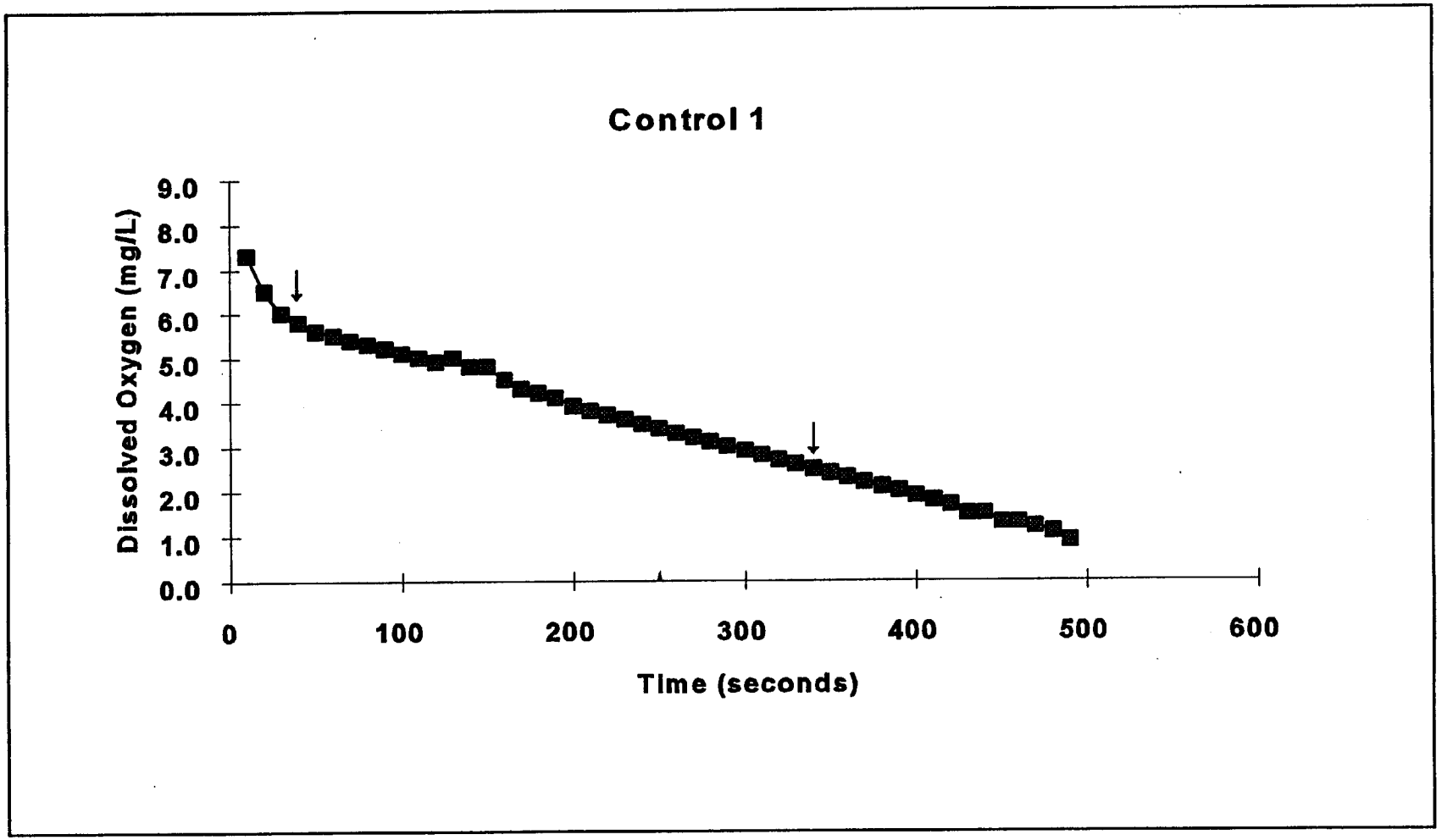

Figure 1. Dissolved Oxygen Concentration Over Time, Control 1. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

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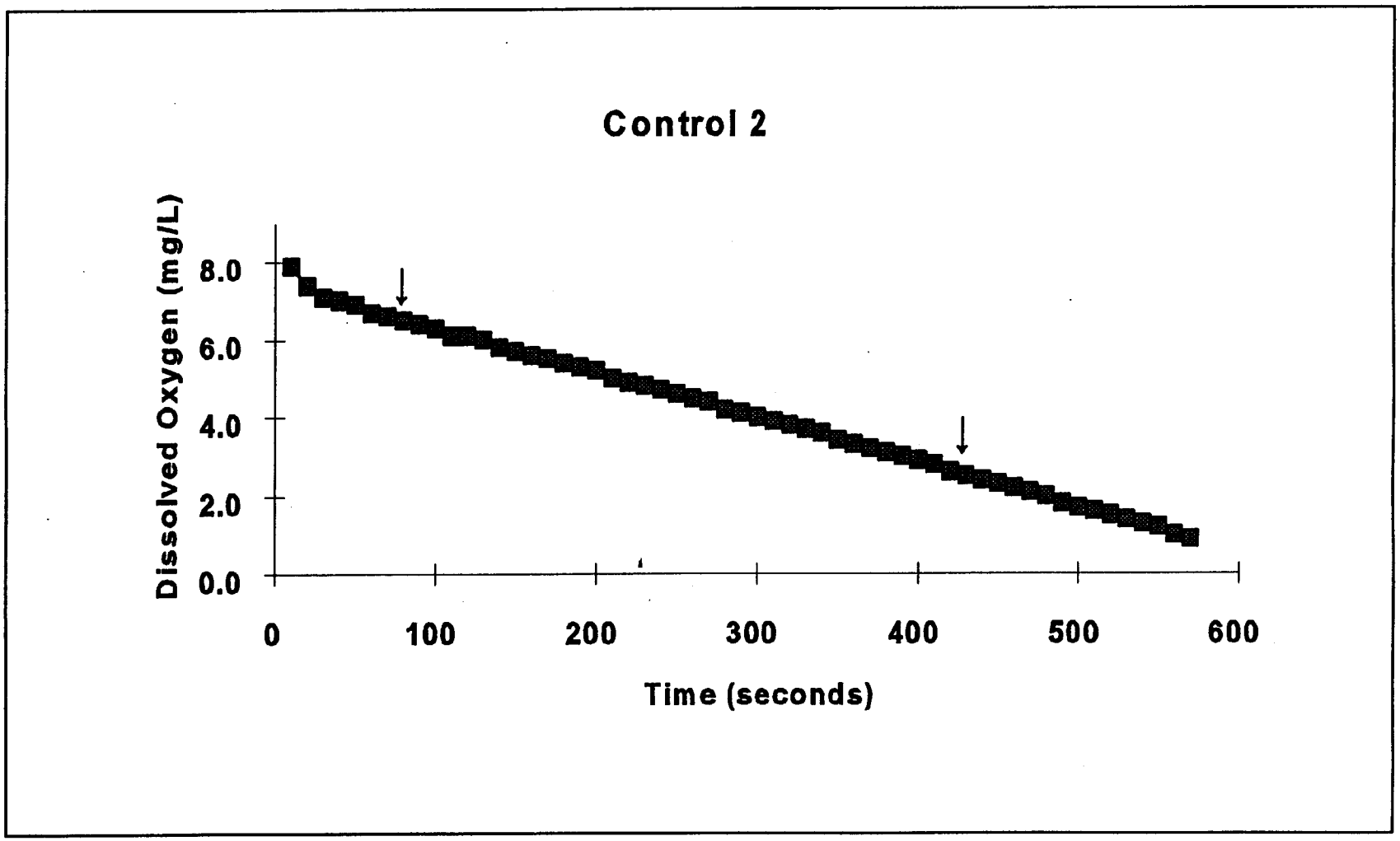

Figure 2. Dissolved Oxygen Concentration Over Time, Control 2. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

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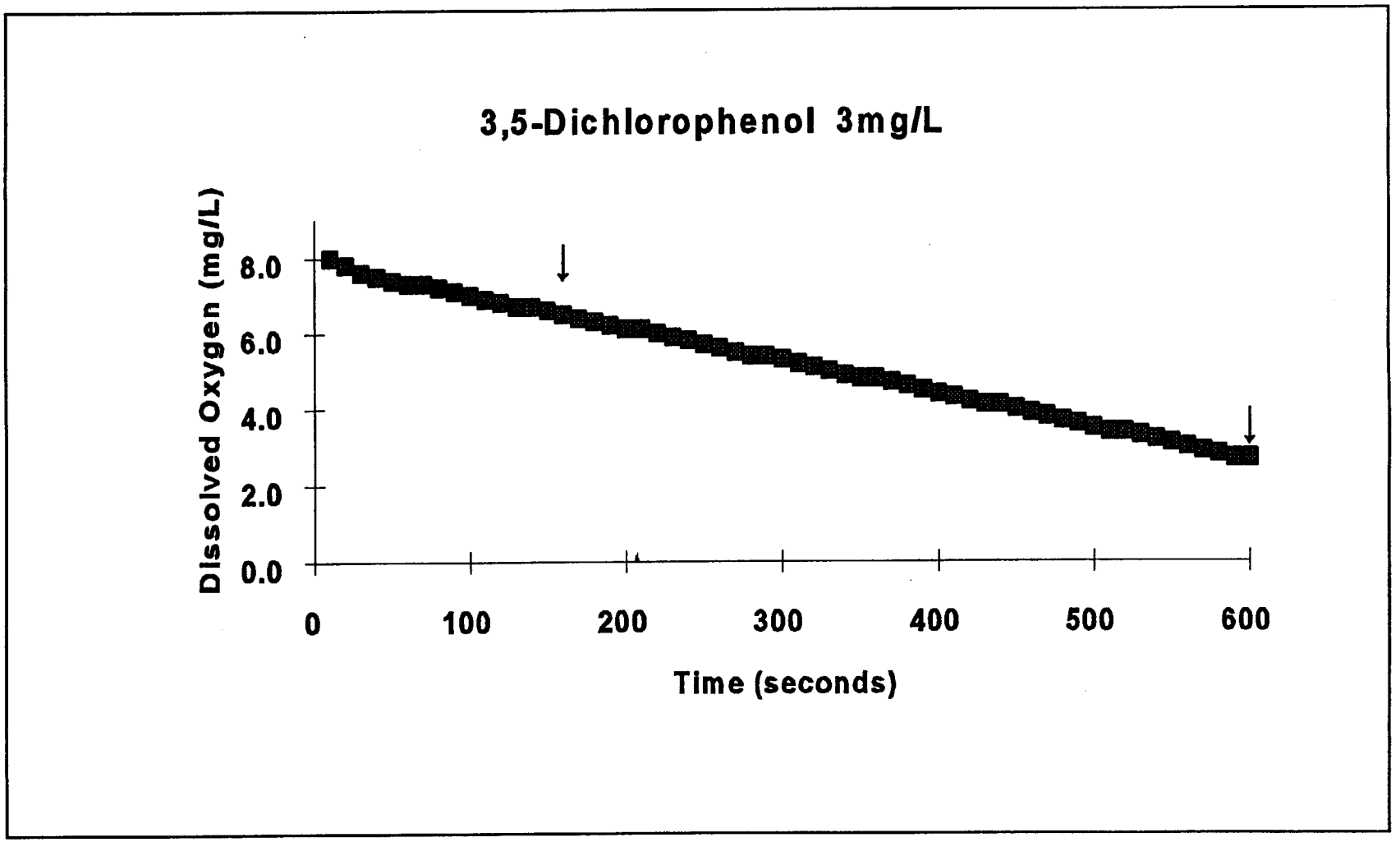

Figure 3. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 3 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

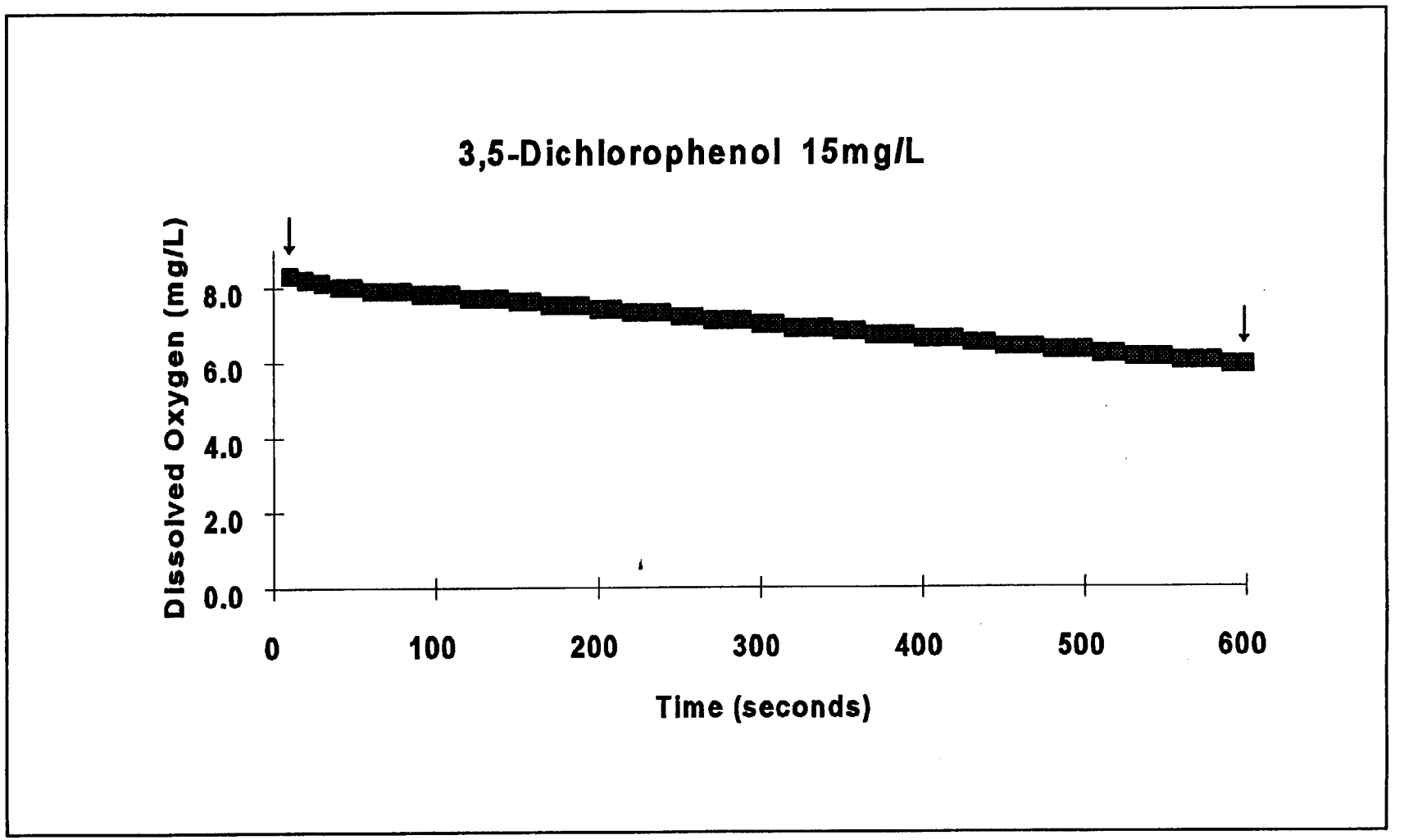

Figure 4. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 15 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

AMENDED

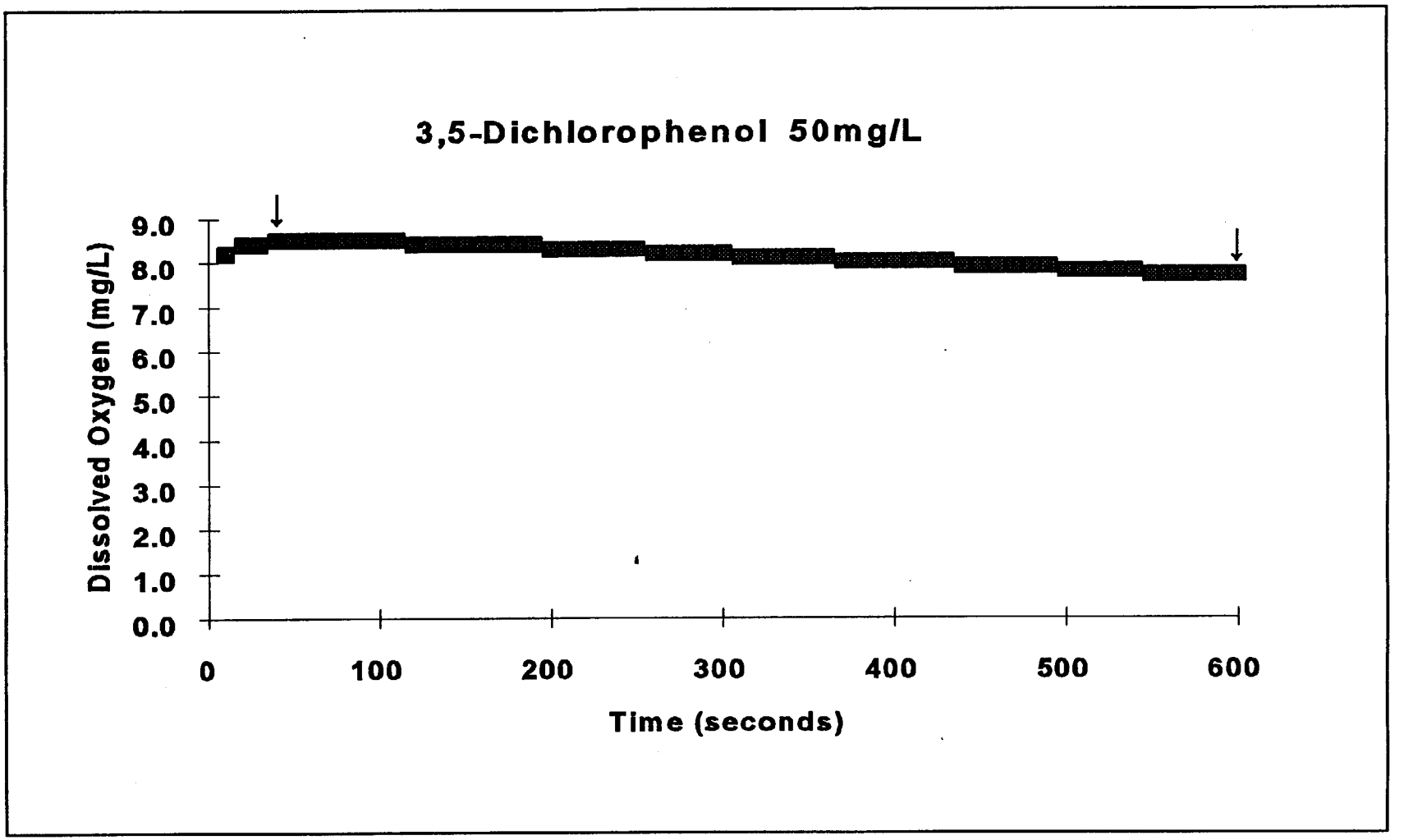

Figure 5. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 50 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

AMENDED

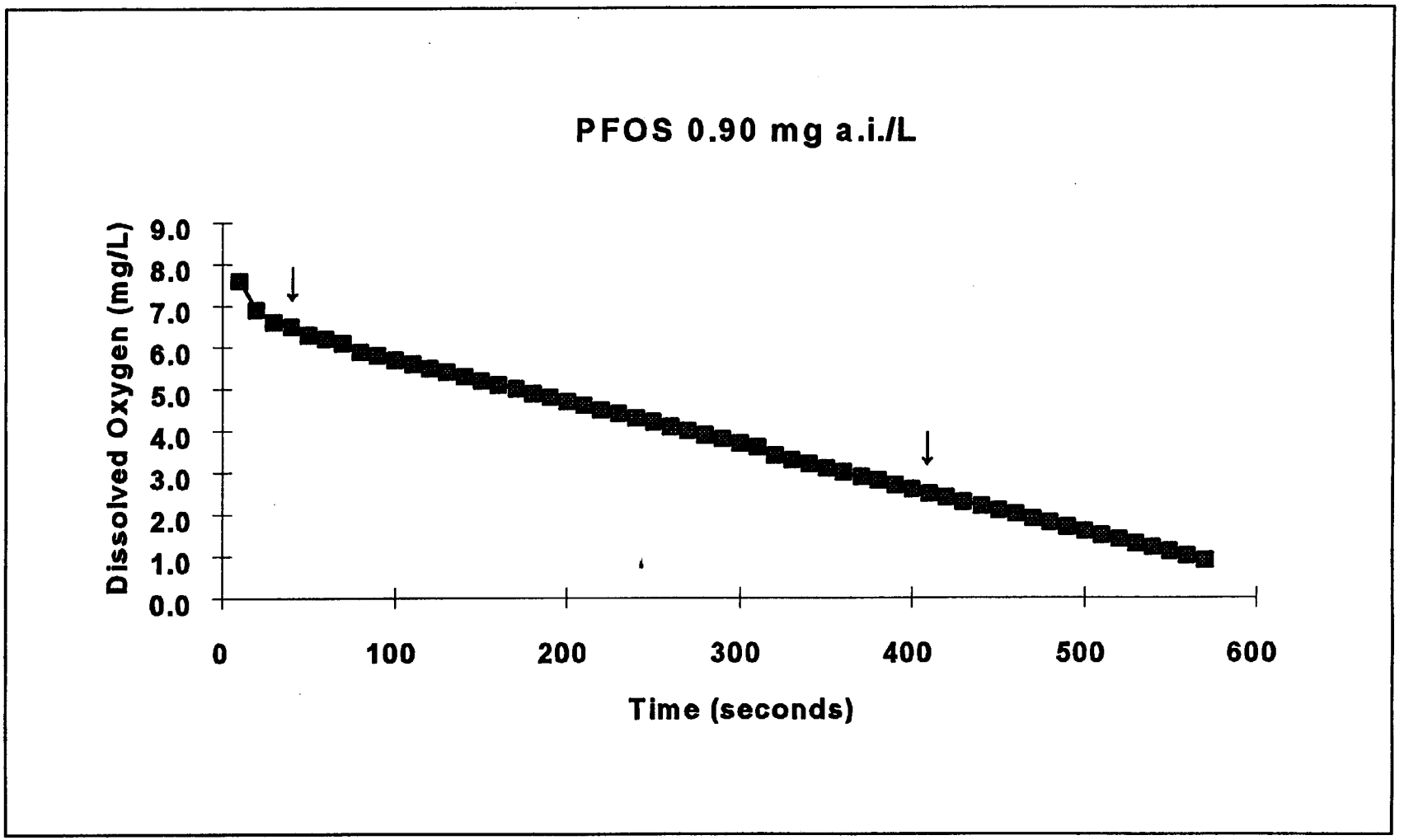

Figure 6. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 0.90 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

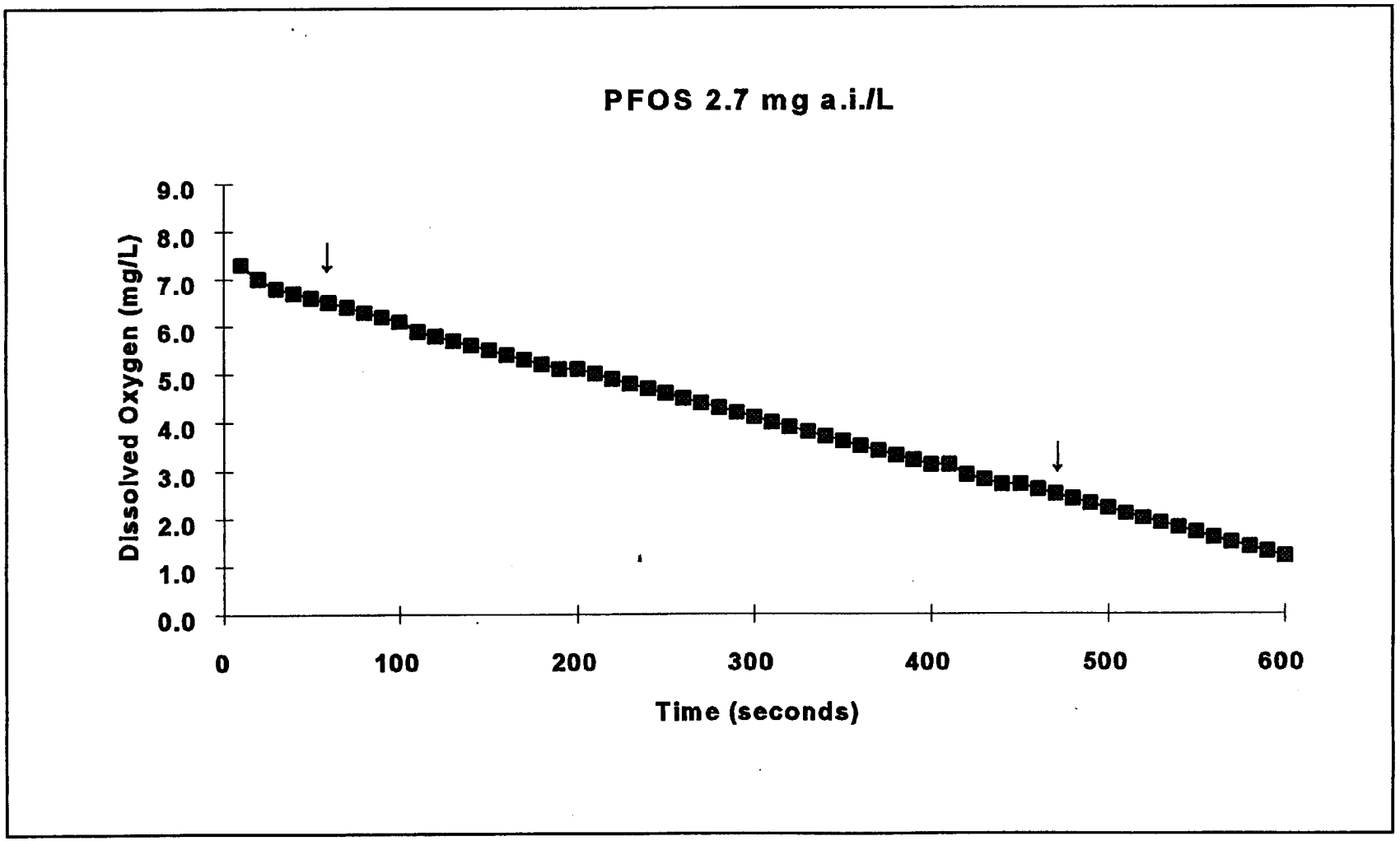

Figure 7. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 2.7 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

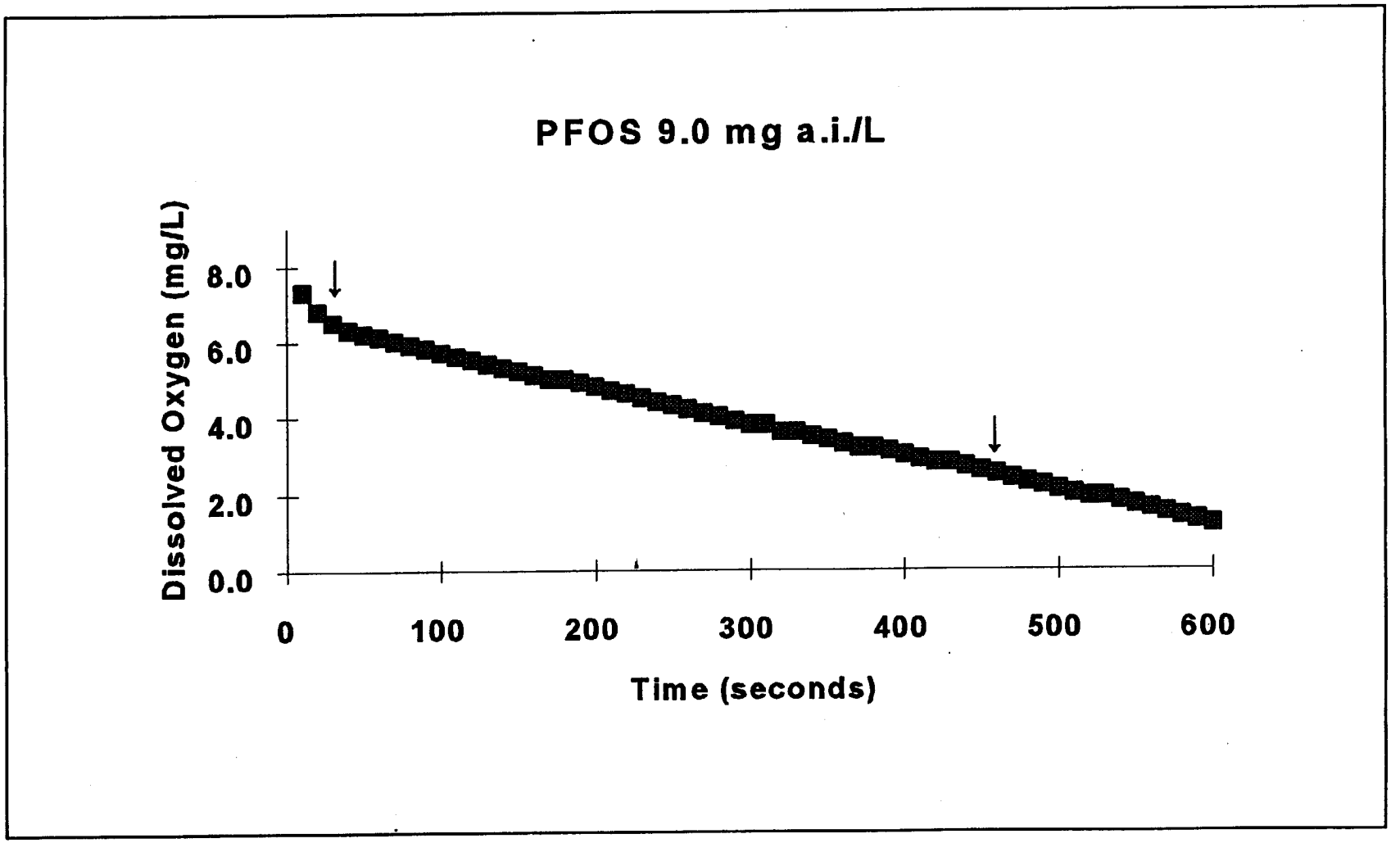

Figure 8. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 9.0 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

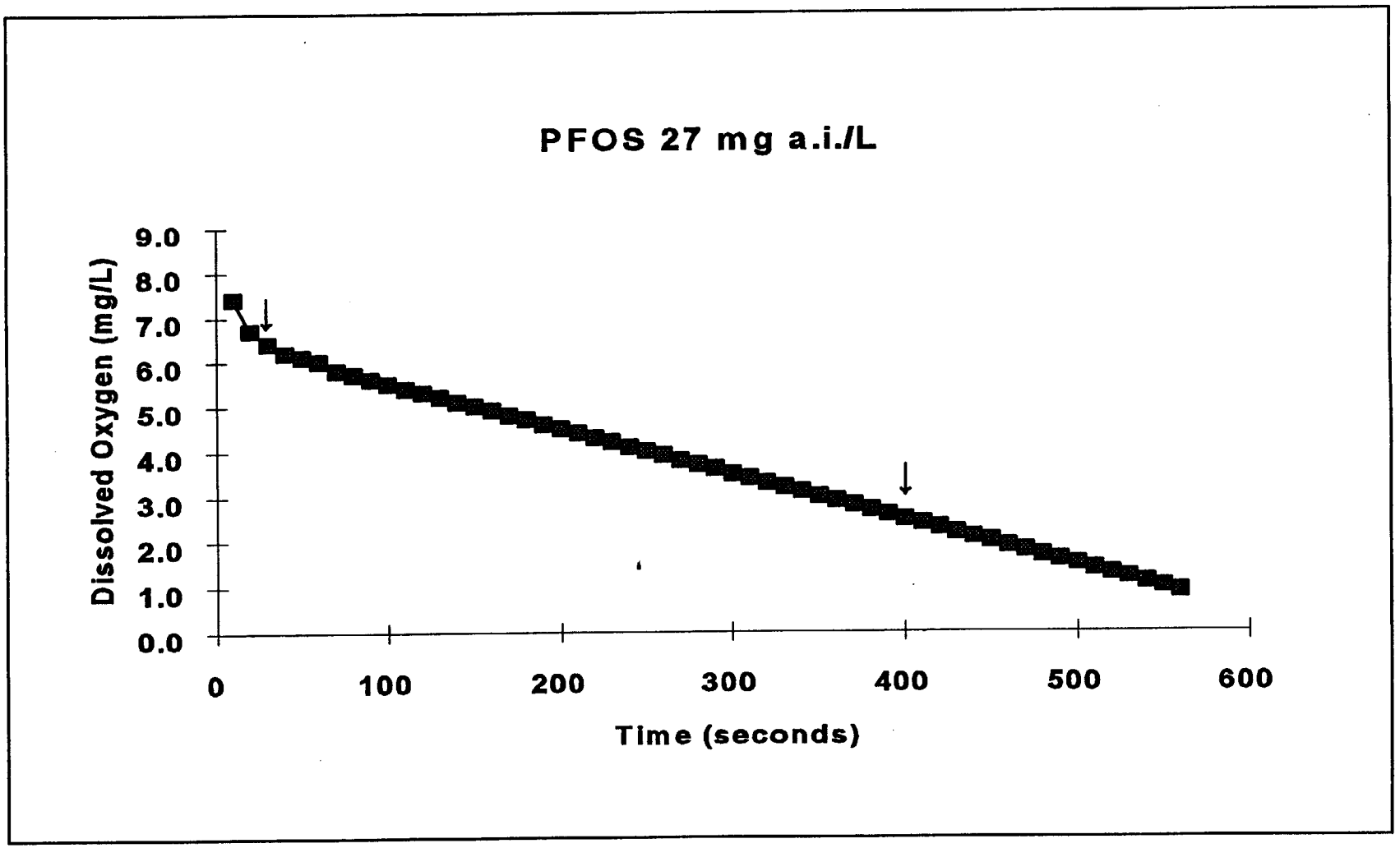

Figure 9. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 27 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

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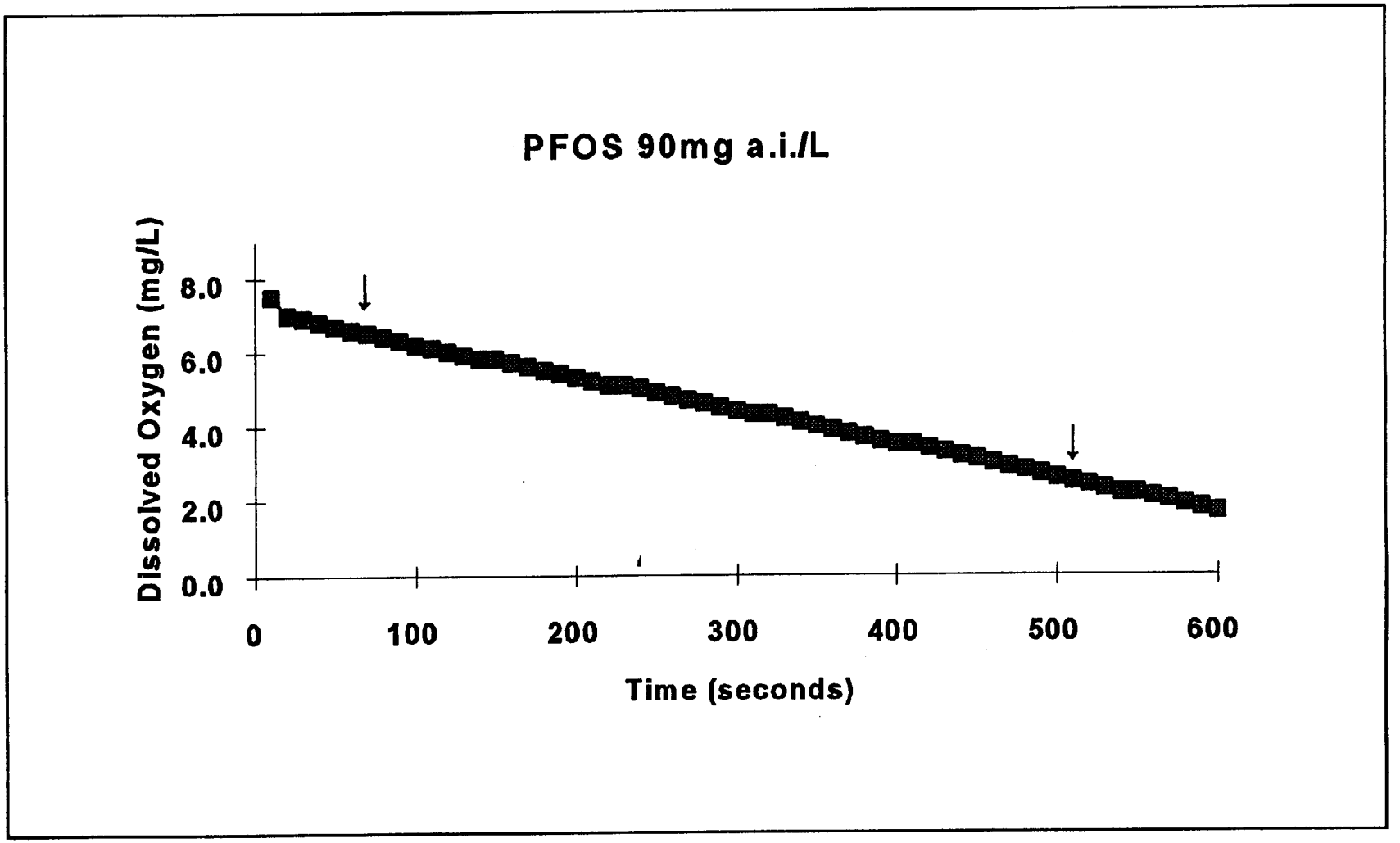

Figure 10. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 90 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

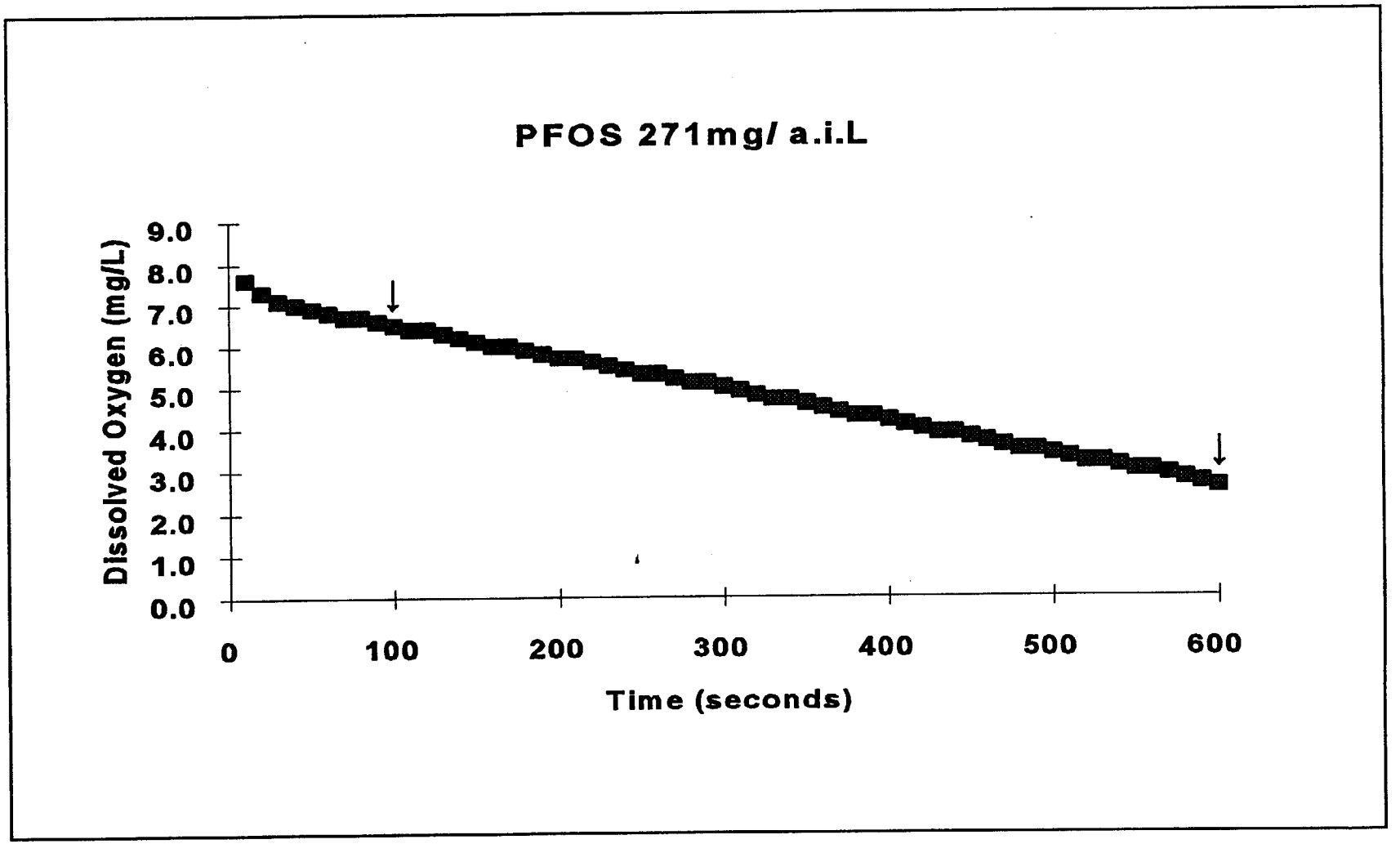

Figure 11. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 271 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate.

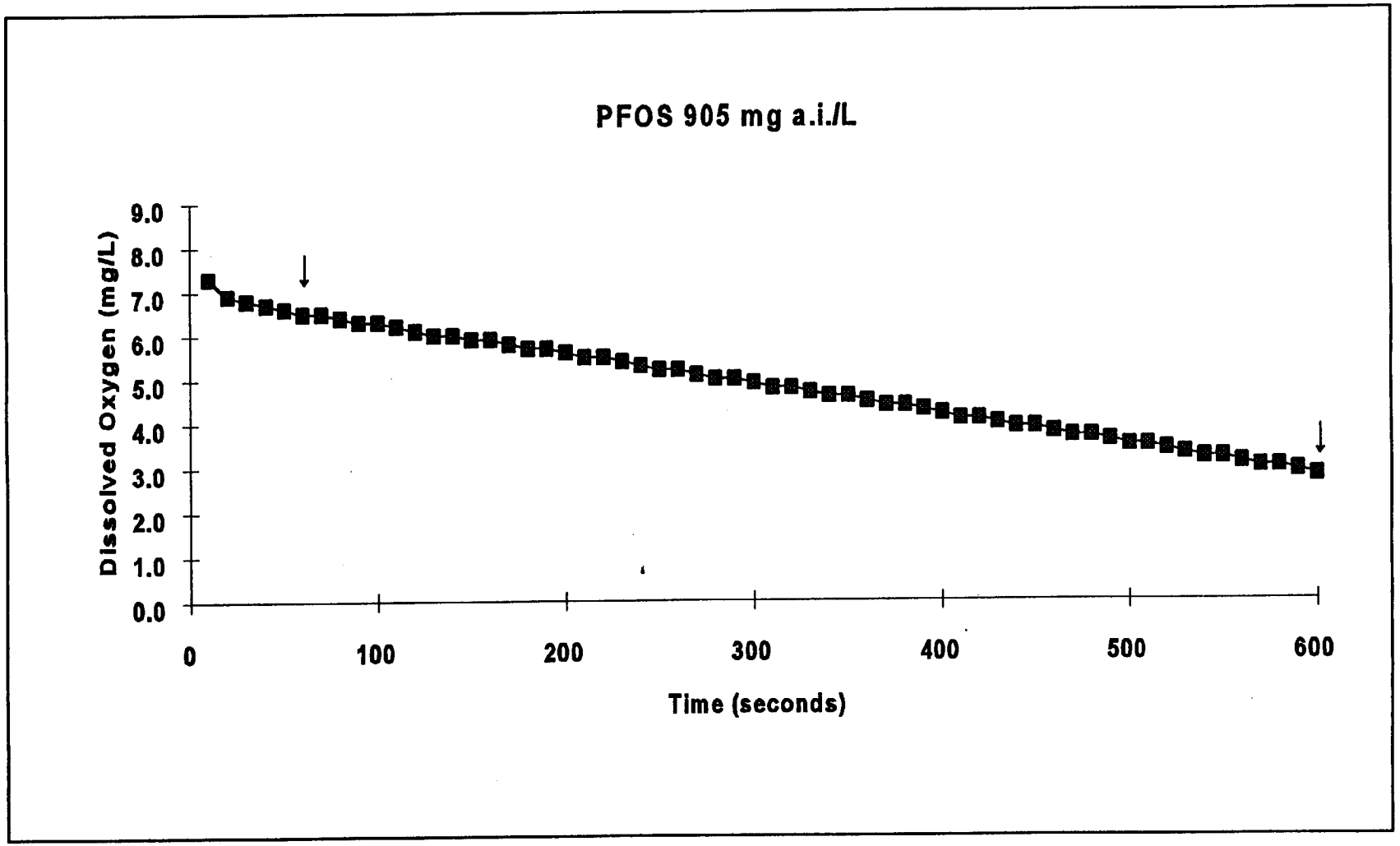

Figure 12. Dissolved Oxygen Concentration Over Time, Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS), 905 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration.

APPENDIX I
Measured Dissolved Oxygen (DO) Concentrations (mg O_2/L)

Time (min.)	Treatment Dissolved Oxygen (mg/L)					
	Control 1	3,5-Dichlorophenol 3 mg/L	3,5-Dichlorophenol 15 mg/L	3,5-Dichlorophenol 50 mg/L	PFOS 0.90 mg a.i./L	PFOS 2.7 mg a.i./L
00:10	7.3	8.0	**8.3**	8.2	7.6	7.3
00:20	6.5	7.8	**8.2**	8.4	6.9	7.0
00:30	6.0	7.6	**8.1**	8.4	6.6	6.8
00:40	**5.8**	7.5	**8.0**	**8.5**	**6.5**	6.7
00:50	**5.6**	7.4	**8.0**	**8.5**	6.3	6.6
01:00	**5.5**	7.3	7.9	**8.5**	6.2	**6.5**
01:10	**5.4**	7.3	7.9	**8.5**	6.1	**6.4**
01:20	**5.3**	7.2	7.9	**8.5**	**5.9**	**6.3**
01:30	**5.2**	7.1	**7.8**	**8.5**	**5.8**	**6.2**
01:40	**5.1**	7.0	**7.8**	**8.5**	**5.7**	**6.1**
01:50	**5.0**	6.9	**7.8**	**8.5**	**5.6**	**5.9**
02:00	**4.9**	6.8	**7.7**	**8.4**	**5.5**	**5.8**
02:10	**5.0**	6.7	**7.7**	**8.4**	**5.4**	**5.7**
02:20	**4.8**	6.7	**7.7**	**8.4**	**5.3**	**5.6**
02:30	**4.8**	6.6	**7.6**	**8.4**	**5.2**	**5.5**
02:40	**4.5**	6.5	**7.6**	**8.4**	**5.1**	**5.4**
02:50	**4.3**	6.4	**7.5**	**8.4**	**5.0**	**5.3**
03:00	**4.2**	6.3	**7.5**	**8.4**	**4.9**	**5.2**
03:10	**4.1**	6.2	**7.5**	**8.4**	**4.8**	**5.1**
03:20	**3.9**	6.1	**7.4**	**8.3**	**4.7**	**5.1**
03:30	**3.8**	6.1	**7.4**	**8.3**	**4.6**	**5.0**
03:40	**3.7**	**6.0**	**7.3**	**8.3**	**4.5**	**4.9**
03:50	**3.6**	**5.9**	**7.3**	**8.3**	**4.4**	**4.8**
04:00	**3.5**	**5.8**	**7.3**	**8.3**	**4.3**	**4.7**
04:10	**3.4**	**5.7**	**7.2**	**8.3**	**4.2**	**4.6**
04:20	**3.3**	**5.6**	**7.2**	**8.2**	**4.1**	**4.5**
04.30	**3.2**	**5.5**	**7.1**	**8.2**	**4.0**	**4.4**
04.40	**3.1**	**5.4**	**7.1**	**8.2**	**3.9**	**4.3**
04.50	**3.0**	**5.4**	**7.1**	**8.2**	**3.8**	**4.2**
05:00	**2.9**	**5.3**	**7.0**	**8.2**	**3.7**	**4.1**
05:10	**2.8**	**5.2**	**7.0**	**8.1**	**3.6**	**4.0**
05:20	**2.7**	**5.1**	**6.9**	**8.1**	**3.4**	**3.9**
05:30	**2.6**	**5.0**	**6.9**	**8.1**	**3.3**	**3.8**
05:40	**2.5**	**4.9**	**6.9**	**8.1**	**3.2**	**3.7**
05:50	2.4	**4.8**	**6.8**	**8.1**	**3.1**	**3.6**
06:00	2.3	**4.8**	**6.8**	**8.1**	**3.0**	**3.5**
06:10	2.2	**4.7**	**6.7**	**8.0**	**2.9**	**3.4**
06:20	2.1	**4.6**	**6.7**	**8.0**	**2.8**	**3.3**
06:30	2.0	**4.5**	**6.7**	**8.0**	**2.7**	**3.2**
06:40	1.9	**4.4**	**6.6**	**8.0**	**2.6**	**3.1**
06:50	1.8	**4.3**	**6.6**	**8.0**	**2.5**	**3.1**
07:00	1.7	**4.2**	**6.6**	**8.0**	2.4	**2.9**
07:10	1.5	**4.1**	**6.5**	**8.0**	2.3	**2.8**
07:20	1.5	**4.1**	**6.5**	**7.9**	2.2	**2.7**
07:30	1.3	**4.0**	**6.4**	**7.9**	2.1	**2.7**
07:40	1.3	**3.9**	**6.4**	**7.9**	2.0	**2.6**
07:50	1.2	**3.8**	**6.4**	**7.9**	1.9	**2.5**
08:00	1.1	**3.7**	**6.3**	**7.9**	1.8	2.4
08:10	0.9	**3.6**	**6.3**	**7.9**	1.7	2.3
08:20	–	**3.5**	**6.3**	**7.8**	1.6	2.2
08:30	–	**3.4**	**6.2**	**7.8**	1.5	2.1
08:40	–	**3.4**	**6.2**	**7.8**	1.4	2.0
08:50	–	**3.3**	**6.1**	**7.8**	1.3	1.9
09:00	–	**3.2**	**6.1**	**7.8**	1.2	1.8
09:10	–	**3.1**	**6.1**	**7.7**	1.1	1.7
09:20	–	**3.0**	**6.0**	**7.7**	1.0	1.6
09:30	–	**2.9**	**6.0**	**7.7**	0.9	1.5
09:40	–	**2.8**	**6.0**	**7.7**	–	1.4
09:50	–	**2.7**	**5.9**	**7.7**	–	1.3
10:00	–	**2.7**	**5.9**	**7.7**	–	1.2

Bold numbers indicate dissolved oxygen concentrations used to calculate respiration rates.

AMENDED

APPENDIX I (Continued)
Measured Dissolved Oxygen (DO) Concentrations (mg O_2/L)

Time (min.)	Treatment Dissolved Oxygen (mg/L)					
	PFOS 9.0 mg a.i./L	PFOS 27 mg a.i./L	PFOS 90 mg a.i./L	PFOS 271 mg a.i./L	PFOS 905 mg a.i./L	Control 2
00:10	7.3	7.4	7.5	7.6	7.3	7.9
00:20	6.8	6.7	7.0	7.3	6.9	7.4
00:30	6.5	6.4	6.9	7.1	6.8	7.1
00:40	6.3	6.2	6.8	7.0	6.7	7.0
00:50	6.2	6.1	6.7	6.9	6.6	6.9
01:00	6.1	6.0	6.6	6.8	6.5	6.7
01:10	6.0	5.8	6.5	6.7	6.5	6.6
01:20	5.9	5.7	6.4	6.7	6.4	6.5
01:30	5.8	5.6	6.3	6.6	6.3	6.4
01:40	5.7	5.5	6.2	6.5	6.3	6.3
01:50	5.6	5.4	6.1	6.4	6.2	6.1
02:00	5.5	5.3	6.0	6.4	6.1	6.1
02:10	5.4	5.2	5.9	6.3	6.0	6.0
02:20	5.3	5.1	5.8	6.2	6.0	5.8
02:30	5.2	5.0	5.8	6.1	5.9	5.7
02:40	5.1	4.9	5.7	6.0	5.9	5.6
02:50	5.0	4.8	5.6	6.0	5.8	5.5
03:00	5.0	4.7	5.5	5.9	5.7	5.4
03:10	4.9	4.6	5.4	5.8	5.7	5.3
03:20	4.8	4.5	5.3	5.7	5.6	5.2
03:30	4.7	4.4	5.2	5.7	5.5	5.0
03:40	4.6	4.3	5.1	5.6	5.5	4.9
03:50	4.5	4.2	5.1	5.5	5.4	4.8
04:00	4.4	4.1	5.0	5.4	5.3	4.7
04:10	4.3	4.0	4.9	5.3	5.2	4.6
04:20	4.2	3.9	4.8	5.3	5.2	4.5
04.30	4.1	3.8	4.7	5.2	5.1	4.4
04.40	4.0	3.7	4.6	5.1	5.0	4.2
04.50	3.9	3.6	4.5	5.1	5.0	4.1
05:00	3.8	3.5	4.4	5.0	4.9	4.0
05:10	3.8	3.4	4.3	4.9	4.8	3.9
05:20	3.6	3.3	4.3	4.8	4.8	3.8
05:30	3.6	3.2	4.2	4.7	4.7	3.7
05:40	3.5	3.1	4.1	4.7	4.6	3.6
05:50	3.4	3.0	4.0	4.6	4.6	3.4
06:00	3.3	2.9	3.9	4.5	4.5	3.3
06:10	3.2	2.8	3.8	4.4	4.4	3.2
06:20	3.2	2.7	3.7	4.3	4.4	3.1
06:30	3.1	2.6	3.6	4.3	4.3	3.0
06:40	3.0	2.5	3.5	4.2	4.2	2.9
06:50	2.9	2.4	3.5	4.1	4.1	2.8
07:00	2.8	2.3	3.4	4.0	4.1	2.6
07:10	2.8	2.2	3.3	3.9	4.0	2.5
07:20	2.7	2.1	3.2	3.9	3.9	2.4
07:30	2.6	2.0	3.1	3.8	3.9	2.3
07:40	2.5	1.9	3.0	3.7	3.8	2.2
07:50	2.4	1.8	2.9	3.6	3.7	2.1
08:00	2.3	1.7	2.8	3.5	3.7	2.0
08:10	2.2	1.6	2.7	3.5	3.6	1.8
08:20	2.1	1.5	2.6	3.4	3.5	1.7
08:30	2.0	1.4	2.5	3.3	3.5	1.6
08:40	1.9	1.3	2.4	3.2	3.4	1.5
08:50	1.9	1.2	2.3	3.2	3.3	1.4
09:00	1.8	1.1	2.2	3.1	3.2	1.3
09:10	1.7	1.0	2.2	3.0	3.2	1.2
09:20	1.6	0.9	2.1	3.0	3.1	1.0
09:30	1.5	–	2.0	2.9	3.0	0.9
09:40	1.4	–	1.9	2.8	3.0	–
09:50	1.3	–	1.8	2.7	2.9	–
10:00	1.2	–	1.7	2.6	2.8	–

Bold numbers indicate dissolved oxygen concentrations used to calculate respiration rates.

AMENDED

APPENDIX II

Graphical Presentation of Percent Inhibition and Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) Concentration

APPENDIX II

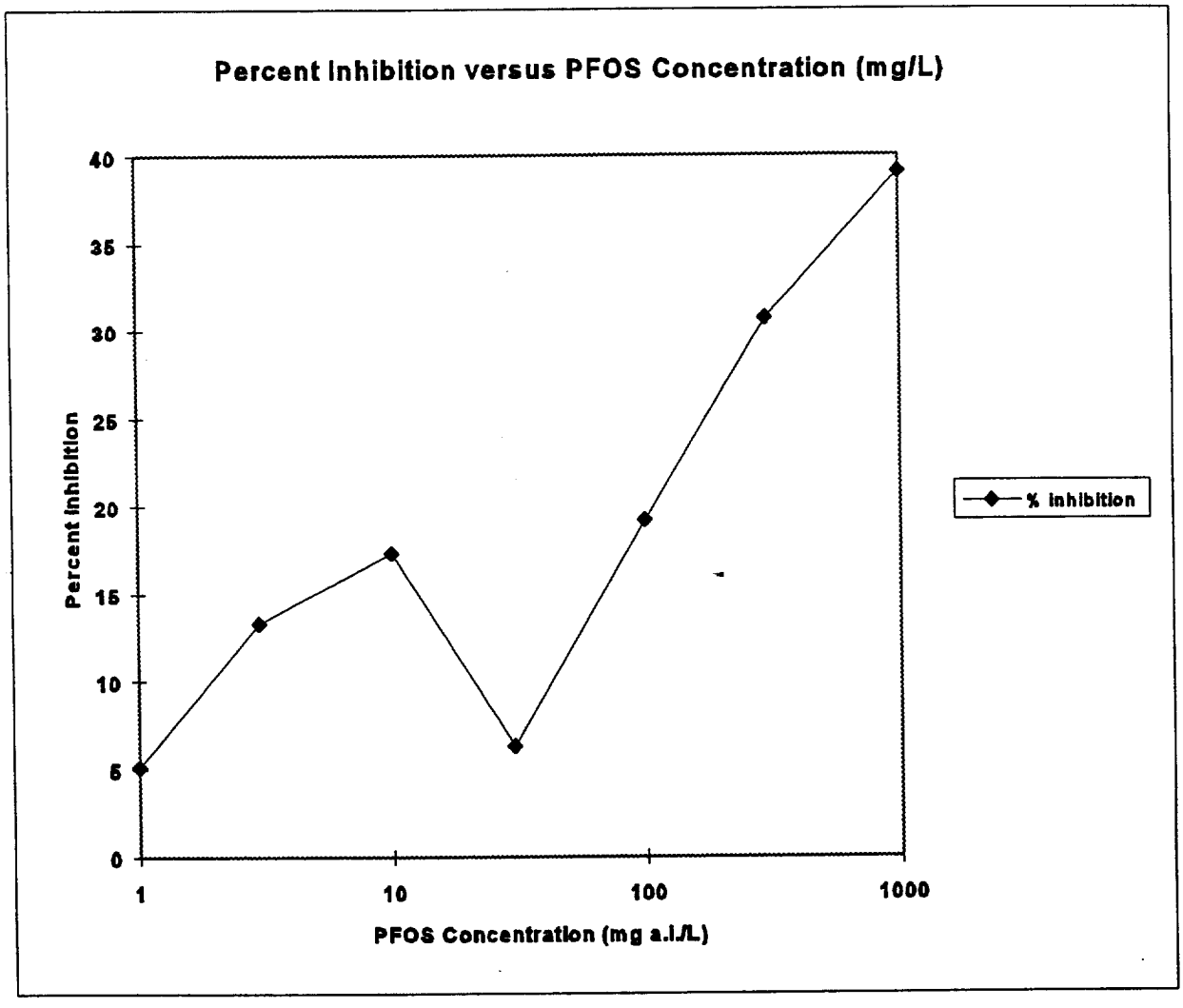

Figure 1. Percent Inhibition versus Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) Concentration

AMENDED

APPENDIX III

Protocol, Amendment and Deviation

APPENDIX III

PROTOCOL

PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

Organisation for Economic Cooperation and Development
OECD Guideline 209

and

Council of European Communities Directive 67/548/EEC
Annex V, Guideline C.11

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

December 1, 1998

APPENDIX III

PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

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:	Ed Schaefer
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Manager of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date:	12/17/98	Experimental Termination Date:	12/17/98
Project No.:	454E-101	Study Room:	No.: 37
Test Concentrations:	1,3,10,30,100,300,1000 mg/L	Int/Date:	CDS 12/11/98
Test Substance No.:	4675	Receipt Date:	10-29-98

PROTOCOL APPROVAL

Edward C. Schaefer STUDY DIRECTOR	12/11/98 DATE
Henry O. Krueger LABORATORY MANAGEMENT	12/10/98 DATE
Susan A. Beach SPONSOR'S REPRESENTATIVE	12/7/98 DATE

PROTOCOL NO.: 454/120198/ASRIT/SUB454 3M LAB REQUEST NO. U2723

APPENDIX III

WILDLIFE INTERNATIONAL LTD.

INTRODUCTION

The purpose of this test is to provide a screening method to identify substances that may adversely affect aerobic microbial treatment plants and to indicate suitable non-inhibitory test substance concentrations for use in biodegradability tests.

OBJECTIVE

The objective of the study will be to assess the effects of the test substance on activated sludge microorganisms by measuring the respiration rate. An EC50 will be calculated, if possible.

EXPERIMENTAL DESIGN

The test will contain a control, reference, and treatment group. The control group is used to determine the background respiration rate of the sludge and will not be exposed to the test substance. The reference group will be dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at concentrations of 3, 15, and 50 mg/L. The test substance will be tested at 1, 3, 10, 30, 100, 300, and 1000 mg/L.

MATERIALS AND METHODS

Test methods are based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1) and Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2).

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP), 40 CFR Part 160.31. The Sponsor is responsible for providing Wildlife International Ltd. written verification that the test substance has been characterized according to GLPs prior to using in the test. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix II) is to be used to provide information necessary for GLP compliance. 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.

APPENDIX III

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.

Stock Solution Preparation

A stock solution of the test substance will be prepared at a nominal concentration of 1000 mg active /L in high quality water (e.g. Nanopure). In addition, a stock solution of 3,5-dichlorophenol will prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with high quality water. While stirring, enough 1N H_2SO_4 (approximately 8 mL) will be added to reach the point of incipient precipitation. The solution of 3,5-dichlorophenol then will be diluted to 1 L with high quality water.

Test Conditions and Apparatus

Control, reference, and treatment test mixtures will be incubated at 20 ± 2°C and aerated for 3 hours at a rate sufficient to maintain solids in suspension. The mixtures will be prepared and aerated in 500 mL plastic Erlenmeyer flasks and then transferred into a 300 mL Biochemical Oxygen Demand (BOD) bottle to the conduct dissolved oxygen (DO) measurements.

Test Inoculum

Activated sludge from the Prospect Bay Wastewater Treatment Plant will be used as the inoculum for the test. The Prospect Bay Facility receives wastes from predominantly domestic sources. The sludge will be sieved using a 2 mm screen and then settled for approximately 30 minutes. The supernatant above the settled solids will be drained and the total suspended solids (TSS) concentration of the settled sludge will be determined. Based on the result, the concentration of the sludge will be adjusted to 4000 mg/L (± 10%) by diluting with municipal water.

If the sludge cannot be used on the day of collection or if the same batch is required to be used on subsequent days (maximum four days), 50 mL of synthetic sewage (Appendix II) will be added to each liter of activated sludge at the end of each working day. The sludge will be aerated overnight at 20 ± 2°C. Before use, the pH and total suspended solids concentration of the activated sludge will be determined and, if necessary, adjusted to pH 6.0 - 8.0 and a solids concentration of 4000 mg/L (± 10%).

APPENDIX III

Procedure

Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate volume of test or reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

Sample Analysis

After three hours of aeration, the contents of the first vessel will be transferred to a BOD bottle and the respiration rate will be measured over a period of up to 10 minutes. Dissolved oxygen readings will be recorded every 10 seconds for 10 minutes or until the DO drops below 1.0 mg/L, which ever comes first. The respiration rate in subsequent vessels will be determined in an identical manner at 15 minute intervals so that the contact time of the test substance with the activated sludge is three hours.

Calculations

A respiration rate will be calculated for each test mixture and expressed in mg O_2/L/hour. The rate will be calculated using DO values between approximately 6.5 mg O_2/L and 2.5 mg O_2/L, or over a 10 minute period if the DO does not reach approximately 2.5 mg O_2/L.

The percent inhibition for each test substance concentration will be calculated using the following equation and plotted against concentration on log paper:

$$\text{Percent Inhibition} = 1 - \frac{2R_s}{RC_1 + RC_2} \times 100$$

APPENDIX III

where

R_s = oxygen consumption rate at a given concentration of the test substance

RC_1 = oxygen consumption rate, Control 1

RC_2 = oxygen consumption rate, Control 2

An EC50 value will be derived, if possible, based on the percent inhibition versus test substance concentration. Confidence limits (95%) for the EC50 will be determined using standard statistical procedures (3).

Quality Control

The test is considered valid only if the following criteria are met:

- the two control respiration rates are within 15% of each other;
- the EC50 (3 hours) of 3,5-dichlorophenol is in the accepted range of 5 to 30 mg/L.

RECORDS TO BE MAINTAINED

Records to be maintained will include, but not limited to, the following:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance as provided by Sponsor.
3. Test initiation and termination dates.
4. Experimental initiation and termination dates.
5. Stock solution concentration calculations and solution preparation.
6. Activated sludge source and pretreatment details.
7. Test temperature and duration.
8. Reference substance results.
9. All dissolved oxygen measurements.
10. Temperature range recorded during test period.
11. Inhibition curve and method for calculation of EC50.
12. If calculated, EC50 and 95% confidence limits.
13. A copy of the final report.

APPENDIX III

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- 7 -

FINAL REPORT

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

1. Name and address of facility performing the study.
2. Dates on which the study was initiated and completed.
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. Identification and characterization of the test substance as provided by Sponsor including name, CAS number, percent active, and other characteristics, if provided by the Sponsor.
6. A description of the transformations and calculations performed on the data.
7. A description of the methods used and reference to any standard method employed.
8. A description of the test system.
9. A description of the preparation of the test solutions, the testing concentration(s), the route of administration, and the duration of the test.
10. A description of all circumstances that may of 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. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
13. The location where the raw data and final report are to be stored.
14. A statement prepared by the Quality Assurance Unit listing the dates that the study inspections and audits were made and the dates of any findings were reported to the Study Director and Management.
15. 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.

APPENDIX III

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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); OECD Principles of Good Laboratory Practices (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. 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.

APPENDIX III

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- 9 -

REFERENCES

1 **Organisation for Economic Cooperation and Development.** 1989. *Activated Sludge Respiration Inhibition Test.* OECD Guideline 209.

2 **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.11, *Activated Sludge Respiration Inhibition Test.*

3 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

APPENDIX III

WILDLIFE INTERNATIONAL LTD.

- 10 -

APPENDIX I
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)

Sample Code or Batch Number: Lot 217

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 been determined prior to the start of this study in accordance with GLP Standards? Yes ___ No X

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient room temperature.

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

Other pertinent stability information:

IV. Toxicity Information:

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

Aquatic: Invertebrate Toxicity (EC/LC50) Fish Toxicity (LC50)

Daphnia magna: 27 mg/L Rainbow trout: 11 mg/L

Daphnia magna: 50 mg/L Fathead minnow: 38 mg/L

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

See MSDS.

APPENDIX III

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APPENDIX II. SYNTHETIC SEWAGE

The synthetic sewage provides the necessary nutrients required for bacterial metabolism. It is prepared by dissolving the following amounts of substances in 1 liter of municipal water:

16.0 g peptone
11.0 g meat extract
3.0 g urea
0.7 g NaCl
0.4 g $CaCl_2 2H_2O$
0.2 g $MgSO_4 7H_2O$
2.8 g K_2HPO_4

Reagent grade chemicals or better will be used when available. The constituents of the synthetic sewage are not known to contain any contaminants that are reasonable expected to be present and are known to be capable of interfering with the study.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

PROTOCOL NO.: 454/120198/ASRIT/SUB454 **AMENDMENT NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454E-101

3M LAB REQUEST NO.: U2723

AMENDMENT: Page 3; Test Substance

Add: The test substance will be administered by direct weight addition. Direct weight addition is the most appropriate route of administration of test substances that are relatively insoluble in water.

REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

AMENDMENT: Page 4; Stock Solution Prepartion

Delete: A stock solution of the test substance will be prepared at a nominal concentration of 1000 mg active/L in high quality water (e.g. Nanopure).

REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

QA Reviewed: LD 12-21-98

APPENDIX III

WILDLIFE INTERNATIONAL LTD.

AMENDMENT: Page 5; Procedure

Change: Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate volume of test or reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

To: Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate amount of test substance or volume of reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

P.C. Schulz — 12/21/98
STUDY DIRECTOR — DATE

[signature] — 2/3/99
LABORATORY MANAGEMENT — DATE

Susan A. Beach — 2/12/99
SPONSOR'S REPRESENTATIVE — DATE

QA Reviewed! LD 12-21-98

APPENDIX III

WILDLIFE INTERNATIONAL LTD.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

PROTOCOL NO.: 454/120198/ASRIT/SUB454 **DEVIATION NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454E-101

DATE OF DEFACTO DEVIATION: December 12, 1998

DEVIATION: Procedure, Page -5-:

The 3 mg/L 3,5-dichlorophenol mixture was aerated for three hours and one minute.

REASON:

Inadvertent error by study personnel.

IMPACT:

This deviation did not impact the integrity of the study.

P.C. Lee Jr. 2-3-99
STUDY DIRECTOR DATE

K. Kim 2/3/99
LABORATORY MANAGEMENT DATE

APPENDIX IV

Report Amendment

1. Original Report: Pages 1-5 and 7

   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 IV.

   Reason: To reflect the issuing of an amended report.

2. Original Report: Page 2

   Amendment: 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 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 reported in mg/L. The certificate of analysis Dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations 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 substance purity of 90.49% at the request of the Sponsor.

AMENDED

APPENDIX IV

- Continued -

Report Amendment

AMENDMENT SIGNATURES:

Edward C. Schaefer
Manager, Biodegradation

4/28/2000
DATE

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

4/28/00
DATE

REVIEWED BY:

Marshall T. Hynson
Quality Assurance Program Supervisor

4/28/2000
DATE

AMENDED

WILDLIFE INTERNATIONAL LTD.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

PROTOCOL NO.: 454/120198/ASRIT/SUB454 **AMENDMENT NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454E-101

3M LAB REQUEST NO.: U2723

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Add: The test substance will be administered by direct weight addition. Direct weight addition is the most appropriate route of administration of test substances that are relatively insoluble in water.

REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

AMENDMENT: Page 4; Stock Solution Prepartion

Delete: A stock solution of the test substance will be prepared at a nominal concentration of 1000 mg active/L in high quality water (e.g. Nanopure).

REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

TA Reviewed! LD 12-21-98

WILDLIFE INTERNATIONAL LTD.

AMENDMENT: Page 5; Procedure

Change: Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate volume of test or reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

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REASON: Route of administration was changed from volumetric addition of an aqueous stock solution to direct weight addition because of the low water solubility of the test substance.

EFFECTIVE DATE: 21 December 1998

P.C. Schlafer — STUDY DIRECTOR — 12/21/98 — DATE

Hy Kuyn — LABORATORY MANAGEMENT — 2/2/99 — DATE

Susan A. Beach — SPONSOR'S REPRESENTATIVE — 2/12/99 — DATE

QA Reviewed! LD 12-21-98

WILDLIFE INTERNATIONAL LTD.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

PROTOCOL NO.: 454/120198/ASRIT/SUB454 **DEVIATION NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454E-101

DATE OF DEFACTO DEVIATION: December 12, 1998

DEVIATION: Procedure, Page -5-:

The 3 mg/L 3,5-dichlorophenol mixture was aerated for three hours and one minute.

REASON:

Inadvertent error by study personnel.

IMPACT:

This deviation did not impact the integrity of the study.

P.C. McLaughlin 2-3-99

STUDY DIRECTOR DATE

LABORATORY MANAGEMENT 2/3/99

DATE

PROTOCOL

PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

Organisation for Economic Cooperation and Development
OECD Guideline 209

and

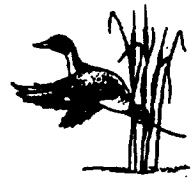
Council of European Communities Directive 67/548/EEC
Annex V, Guideline C.11

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

December 1, 1998

PFOS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

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:	Ed Schaefer
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Manager of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date:	12/17/98	Experimental Termination Date:	12/17/98
Project No.:	454E-101	Study Room:	No: 37
Test Concentrations:	1,3,10,30,100,300,1000 mg/L	Int/Date:	EDS 12/11/98
Test Substance No.:	4675	Receipt Date:	10-29-98

PROTOCOL APPROVAL

STUDY DIRECTOR	DATE 12/11/98
LABORATORY MANAGEMENT	DATE 12/10/98
SPONSOR'S REPRESENTATIVE	DATE 12/7/98

INTRODUCTION

The purpose of this test is to provide a screening method to identify substances that may adversely affect aerobic microbial treatment plants and to indicate suitable non-inhibitory test substance concentrations for use in biodegradability tests.

OBJECTIVE

The objective of the study will be to assess the effects of the test substance on activated sludge microorganisms by measuring the respiration rate. An EC50 will be calculated, if possible.

EXPERIMENTAL DESIGN

The test will contain a control, reference, and treatment group. The control group is used to determine the background respiration rate of the sludge and will not be exposed to the test substance. The reference group will be dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at concentrations of 3, 15, and 50 mg/L. The test substance will be tested at 1, 3, 10, 30, 100, 300, and 1000 mg/L.

MATERIALS AND METHODS

Test methods are based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1) and Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2).

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP), 40 CFR Part 160.31. The Sponsor is responsible for providing Wildlife International Ltd. written verification that the test substance has been characterized according to GLPs prior to using in the test. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix II) is to be used to provide information necessary for GLP compliance. 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 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.

Stock Solution Preparation

A stock solution of the test substance will be prepared at a nominal concentration of 1000 mg active /L in high quality water (e.g. Nanopure). In addition, a stock solution of 3,5-dichlorophenol will prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with high quality water. While stirring, enough 1N H_2SO_4 (approximately 8 mL) will be added to reach the point of incipient precipitation. The solution of 3,5-dichlorophenol then will be diluted to 1 L with high quality water.

Test Conditions and Apparatus

Control, reference, and treatment test mixtures will be incubated at 20 ± 2°C and aerated for 3 hours at a rate sufficient to maintain solids in suspension. The mixtures will be prepared and aerated in 500 mL plastic Erlenmeyer flasks and then transferred into a 300 mL Biochemical Oxygen Demand (BOD) bottle to the conduct dissolved oxygen (DO) measurements.

Test Inoculum

Activated sludge from the Prospect Bay Wastewater Treatment Plant will be used as the inoculum for the test. The Prospect Bay Facility receives wastes from predominantly domestic sources. The sludge will be sieved using a 2 mm screen and then settled for approximately 30 minutes. The supernatant above the settled solids will be drained and the total suspended solids (TSS) concentration of the settled sludge will be determined. Based on the result, the concentration of the sludge will be adjusted to 4000 mg/L (± 10%) by diluting with municipal water.

If the sludge cannot be used on the day of collection or if the same batch is required to be used on subsequent days (maximum four days), 50 mL of synthetic sewage (Appendix II) will be added to each liter of activated sludge at the end of each working day. The sludge will be aerated overnight at 20 ± 2°C. Before use, the pH and total suspended solids concentration of the activated sludge will be determined and, if necessary, adjusted to pH 6.0 - 8.0 and a solids concentration of 4000 mg/L (± 10%).

Procedure

Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate volume of test or reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

Sample Analysis

After three hours of aeration, the contents of the first vessel will be transferred to a BOD bottle and the respiration rate will be measured over a period of up to 10 minutes. Dissolved oxygen readings will be recorded every 10 seconds for 10 minutes or until the DO drops below 1.0 mg/L, which ever comes first. The respiration rate in subsequent vessels will be determined in an identical manner at 15 minute intervals so that the contact time of the test substance with the activated sludge is three hours.

Calculations

A respiration rate will be calculated for each test mixture and expressed in mg O_2/L/hour. The rate will be calculated using DO values between approximately 6.5 mg O_2/L and 2.5 mg O_2/L, or over a 10 minute period if the DO does not reach approximately 2.5 mg O_2/L.

The percent inhibition for each test substance concentration will be calculated using the following equation and plotted against concentration on log paper:

$$Percent\ Inhibition = 1 - \frac{2R_s}{RC_1 + RC_2} \times 100$$

where

R_s = oxygen consumption rate at a given concentration of the test substance

RC_1 = oxygen consumption rate, Control 1

RC_2 = oxygen consumption rate, Control 2

An EC50 value will be derived, if possible, based on the percent inhibition versus test substance concentration. Confidence limits (95%) for the EC50 will be determined using standard statistical procedures (3).

Quality Control

The test is considered valid only if the following criteria are met:

- the two control respiration rates are within 15% of each other;
- the EC50 (3 hours) of 3,5-dichlorophenol is in the accepted range of 5 to 30 mg/L.

RECORDS TO BE MAINTAINED

Records to be maintained will include, but not limited to, the following:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance as provided by Sponsor.
3. Test initiation and termination dates.
4. Experimental initiation and termination dates.
5. Stock solution concentration calculations and solution preparation.
6. Activated sludge source and pretreatment details.
7. Test temperature and duration.
8. Reference substance results.
9. All dissolved oxygen measurements.
10. Temperature range recorded during test period.
11. Inhibition curve and method for calculation of EC50.
12. If calculated, EC50 and 95% confidence limits.
13. A copy of the final report.

FINAL REPORT

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

1. Name and address of facility performing the study.
2. Dates on which the study was initiated and completed.
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. Identification and characterization of the test substance as provided by Sponsor including name, CAS number, percent active, and other characteristics, if provided by the Sponsor.
6. A description of the transformations and calculations performed on the data.
7. A description of the methods used and reference to any standard method employed.
8. A description of the test system.
9. A description of the preparation of the test solutions, the testing concentration(s), the route of administration, and the duration of the test.
10. A description of all circumstances that may of 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. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
13. The location where the raw data and final report are to be stored.
14. A statement prepared by the Quality Assurance Unit listing the dates that the study inspections and audits were made and the dates of any findings were reported to the Study Director and Management.
15. 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); OECD Principles of Good Laboratory Practices (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. 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 **Organisation for Economic Cooperation and Development.** 1989. *Activated Sludge Respiration Inhibition Test.* OECD Guideline 209.

2 **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.11, *Activated Sludge Respiration Inhibition Test.*

3 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

APPENDIX I
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS)

Sample Code or Batch Number: Lot 217

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 been determined prior to the start of this study in accordance with GLP Standards? Yes ___ No X

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient room temperature.

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

Other pertinent stability information:

IV. Toxicity Information:

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

Aquatic:

Invertebrate Toxicity (EC/LC50)	Fish Toxicity (LC50)
Daphnia magna: 27 mg/L	Rainbow trout: 11 mg/L
Daphnia magna: 50 mg/L	Fathead minnow: 38 mg/L

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

See MSDS.

APPENDIX II. SYNTHETIC SEWAGE

The synthetic sewage provides the necessary nutrients required for bacterial metabolism. It is prepared by dissolving the following amounts of substances in 1 liter of municipal water:

16.0 g peptone
11.0 g meat extract
3.0 g urea
0.7 g NaCl
0.4 g $CaCl_2$ $2H_2O$
0.2 g $MgSO_4$ $7H_2O$
2.8 g K_2HPO_4

Reagent grade chemicals or better will be used when available. The constituents of the synthetic sewage are not known to contain any contaminants that are reasonable expected to be present and are known to be capable of interfering with the study.