22) OECD 209-OPPTS 850.6800, Modified activated sludge respiration inhibition test, 454E-102A

PFBS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454E-102A

3M ENVIRONMENTAL LAB PROJECT NO. E00-1429

Organisation for Economic Cooperation and Development
OECD Guideline 209

and

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

AUTHORS:

Edward C. Schaefer
Abul I. Siddiqui

STUDY INITIATION DATE: November 17, 2000

STUDY COMPLETION DATE: March 14, 2001

SUBMITTED TO:

3M Corporation
Environmental Laboratory
935 Bush Avenue
St. Paul, Minnesota 55144

Wildlife International, Ltd.

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

SANITIZED

DEC 09 2003

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFBS: An Activated Sludge, Respiration Inhibition Test

3M ENVIRONMENTAL LAB PROJECT NO. E00-1429

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454E-102A

STUDY COMPLETION: March 14, 2001

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in EPA 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17), 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.

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

The stability of the test and reference substances 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

3/14/01
DATE

SPONSOR:

3/22/01

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice as published by the U.S. Environmental Protection Agency in EPA 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); 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:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: STUDY DIRECTOR:	MANAGEMENT
Initial Trial: 454E-102			
Test Substance Preparation	November 28, 2000	November 28, 2000	December 6, 2000
D.O. Measurements	November 28, 2000	November 28, 2000	November 29, 2000
Definitive Trial: 454E-102A			
D.O. Measurements	November 30, 2000	November 30, 2000	January 5, 2001
Data and Draft Report	January 5 & 8, 2001	January 8, 2001	January 12, 2001
Final Report	March 14, 2001	March 14, 2001	March 14, 2001

Robert N. McGee, B.S.
Quality Assurance Representative

March 14, 2001
DATE

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFBS: An Activated Sludge, Respiration Inhibition Test

3M ENVIRONMENTAL LAB PROJECT NO. E00-1429

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454E-102A

STUDY DIRECTOR:

Edward C. Schaefer, B.S. DATE 3/14/01
Manager, Biodegradation

MANAGEMENT:

Henry O. Krueger, Ph.D. DATE 3/14/01
Director, Aquatic Toxicology and Non-Target Plants

STUDY INFORMATION

Study Initiation Date:	November 17, 2000
Experimental Start Date:	November 27, 2000
Experimental Termination Date:	November 30, 2000
Study Completion Date:	March 14, 2001

Study Director: Edward C. Schaefer B.S.

Sponsor: 3M Corporation
Environmental Laboratory
935 Bush Avenue
St. Paul, Minnesota 55144

Sponsor's Representative:

Study Personnel: Edward C. Schaefer, B.S., Manager, Biodegradation
Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
Abul Siddiqui, B.A., Scientist, Biodegradation
R. Scott Flaggs, B.S., Biologist, Biodegradation
Rebecca Bryan, B.S., Biologist, Aquatics

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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 concentrations of 1, 3, 10, 30, 100, 300 and 1000 mg a.i./L. After an exposure period of three hours, the respiration rates of the test solutions were measured using a dissolved oxygen meter. The respiration rates in the two controls were 43.6 and 45.9 mg O_2/L/hr, or a difference of 5.0%. 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 15.6 mg /L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test. The EC50 for the test substance was over 1000 mg a.i./L.

Differences in O_2 respiration rates between the control group and test substance treatments were not observed at all test substance concentrations. Following is a summary of the results.

Treatment/Nominal Concentration	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	43.6	NA
Control 2	45.9	NA
3,5-dichlorophenol 3 mg/L	42.4	5.3
3,5-dichlorophenol 15 mg/L	24.4	45.5
3,5-dichlorophenol 50 mg/L	4.3	90.4
Perfluorobutanesulfonate (PFBS) 1 mg a.i./L	45.8	-2.3
Perfluorobutanesulfonate (PFBS) 3 mg a.i./L	45.0	-0.6
Perfluorobutanesulfonate (PFBS) 10 mg a.i./L	40.0	11.0
Perfluorobutanesulfonate (PFBS) 30 mg a.i./L	37.9	15.3
Perfluorobutanesulfonate (PFBS) 100 mg a.i./L	47.6	-6.4
Perfluorobutanesulfonate (PFBS) 300 mg a.i./L	36.9	17.5
Perfluorobutanesulfonate (PFBS) 1000 mg a.i./L	41.1	8.2

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.

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 the original final report are filed under Project Number 454E-102A in the archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to assess the effects of PFBS 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 1, 3, 10, 30, 100, 300 and 1000 mg a.i./L.

MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, "PFBS: An Activated Sludge, Respiration Inhibition Test," (Appendix III). The protocol was based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1), Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2) and Fate, Transport and Transformation Test Guidelines, OPPTS 850.6800 (3).

Test Substance

The test substance, PFBS, was received from 3M Corporation at Wildlife International, Ltd. on June 28, 2000 and was assigned Wildlife International, Ltd. identification number 5292.

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

Name:	Potassium, Perfluorobutane sulfonate (PFBS)
Lot Number:	2
CAS Number:	29420-49-3
Physical Description:	White powder
Expiration Date:	March 2010
Purity:	97.9%
Water Solubility:	Moderate
Storage Conditions:	Ambient room temperature

On the day of administration, stock solutions of the test substance were prepared in NANOpure® at nominal concentrations of 1000 and 2000 mg a.i./L. The test substance was administered by volumetric addition.

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 reference substance was administered by volumetric addition. Following is a description of the reference substance used in this study.

Name:	3,5-dichlorophenol
Manufacturer:	Aldrich Chemical Co., Milwaukee, WI
Lot Number:	02611ES
Physical Description:	White solid
Handling Precautions:	Standard laboratory precautions
Date Received:	January 24, 2000

Expiration Date:	January 24, 2005
Purity:	99.1%
Storage Conditions:	Ambient
CAS Number:	591-35-5
Wildlife International Ltd. ID:	5179

Test Conditions and Apparatus

Control, reference, and treatment test mixtures were incubated at 20 ± 2°C and aerated for three hours at a rate sufficient to provide aerobic conditions and maintain solids in suspension. The mixtures were prepared and aerated in 500 mL plastic Erlenmeyer flasks and then transferred into 300 mL biochemical oxygen demand (BOD) bottles to conduct the dissolved oxygen (DO) measurements.

Test Inoculum

Activated sludge was collected from the Denton Wastewater Treatment Plant Denton Maryland on November 27, 2000. The Denton facility receives wastes from predominately domestic sources. The sludge was sieved using a 2 mm screen and the total suspended solids (TSS) concentration of the sieved sludge was determined. The solids concentration of sludge was adjusted to 4000 mg/L (± 10%) by settling the sludge for approximately 30 minutes and then removing supernatant from above the settled sludge solids.

The sludge was maintained in the laboratory for 3 days prior to use. Approximately 50 mL of synthetic sewage (Protocol, Appendix I) was added to each liter of activated sludge and the sludge was continuously aerated. 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 NANOpure® 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 NANOpure® 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:

$$\textit{Respiration Rate} = \textit{(initial DO – final DO)/(final time – initial time)}$$

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):

$$\textit{Percent Inhibition} = 1 - \frac{2R_s}{RC_1 + RC_2} \; X\,100$$

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 (4). 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 (5, 6, 7). In this study, the probit method was used to calculate the EC50 for the reference group.

RESULTS AND DISCUSSION

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 recorded during the maintenance of the sludge prior to the test was 21°C. The temperature range recorded the day of the test was 21-22°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 3647 mg/L and 7.7, respectively.

The respiration rates in the two controls were 43.6 and 45.9 mg O_2/L/hr, or a difference of 5.0%. The validity of the test was further supported by the results from the 3,5-dichlorophenol reference group, which resulted in an EC50 of 15.6 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 13.1 and 18.5 mg/L.

The inhibitory effects upon respiration at the test substance concentrations analyzed (1, 3, 10, 30, 100, 300 and 1000 mg a.i./L) ranged from –6.4% to 17.5%. Respiration rates of the test substance concentrations evaluated ranged from 36.9 mg O_2/L/hr (at 300 mg a.i./L) to 47.6 mg O_2/L/hr (at 100 mg a.i./L).

CONCLUSION

The maximum inhibitory effect upon respiration from PFBS was observed at the test substance concentration of 300 mg a.i./L, but only reached 17.5%. The EC50 is therefore greater than 1000 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. **Fate, Transport and Transformation Guidelines.** 1996. OPPTS 850-6800, Modified Activated Sludge, Respiration Inhibition Test for Sparingly Soluble Chemicals.

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

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

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

7. **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/Nominal Concentration	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	43.6	NA
Control 2	45.9	NA
3,5-dichlorophenol 3 mg/L	42.4	5.3
3,5-dichlorophenol 15 mg/L	24.4	45.5
3,5-dichlorophenol 50 mg/L	4.3	90.4
Perfluorobutanesulfonate (PFBS) 1 mg a.i./L	45.8	-2.3
Perfluorobutanesulfonate (PFBS) 3 mg a.i./L	45.0	-0.6
Perfluorobutanesulfonate (PFBS) 10 mg a.i./L	40.0	11.0
Perfluorobutanesulfonate (PFBS) 30 mg a.i./L	37.9	15.3
Perfluorobutanesulfonate (PFBS) 100 mg a.i./L	47.6	-6.4
Perfluorobutanesulfonate (PFBS) 300 mg a.i./L	36.9	17.5
Perfluorobutanesulfonate (PFBS) 1000 mg a.i./L	41.1	8.2

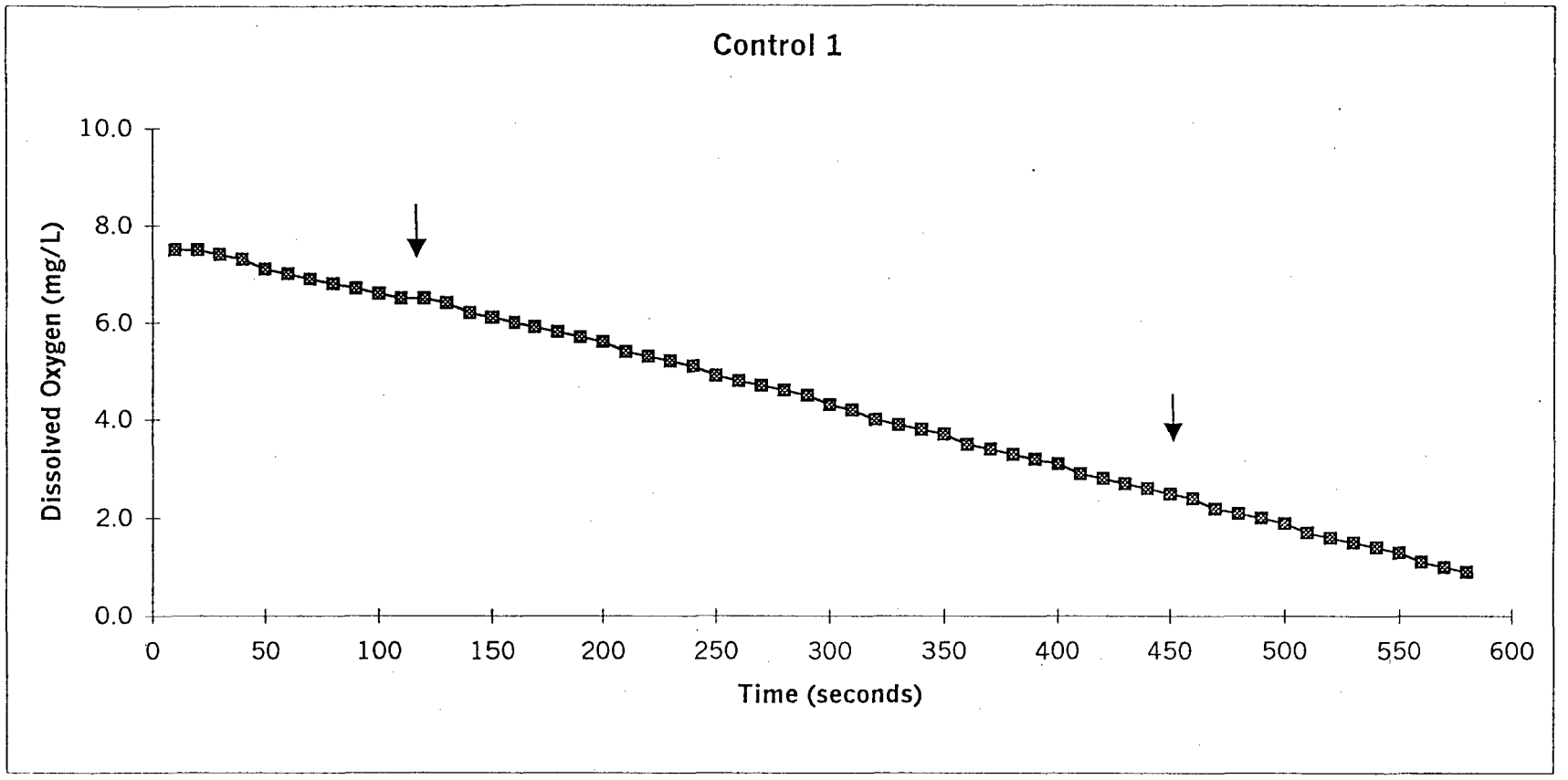

Figure 1. Dissolved Oxygen Concentration Over Time, Control 1. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

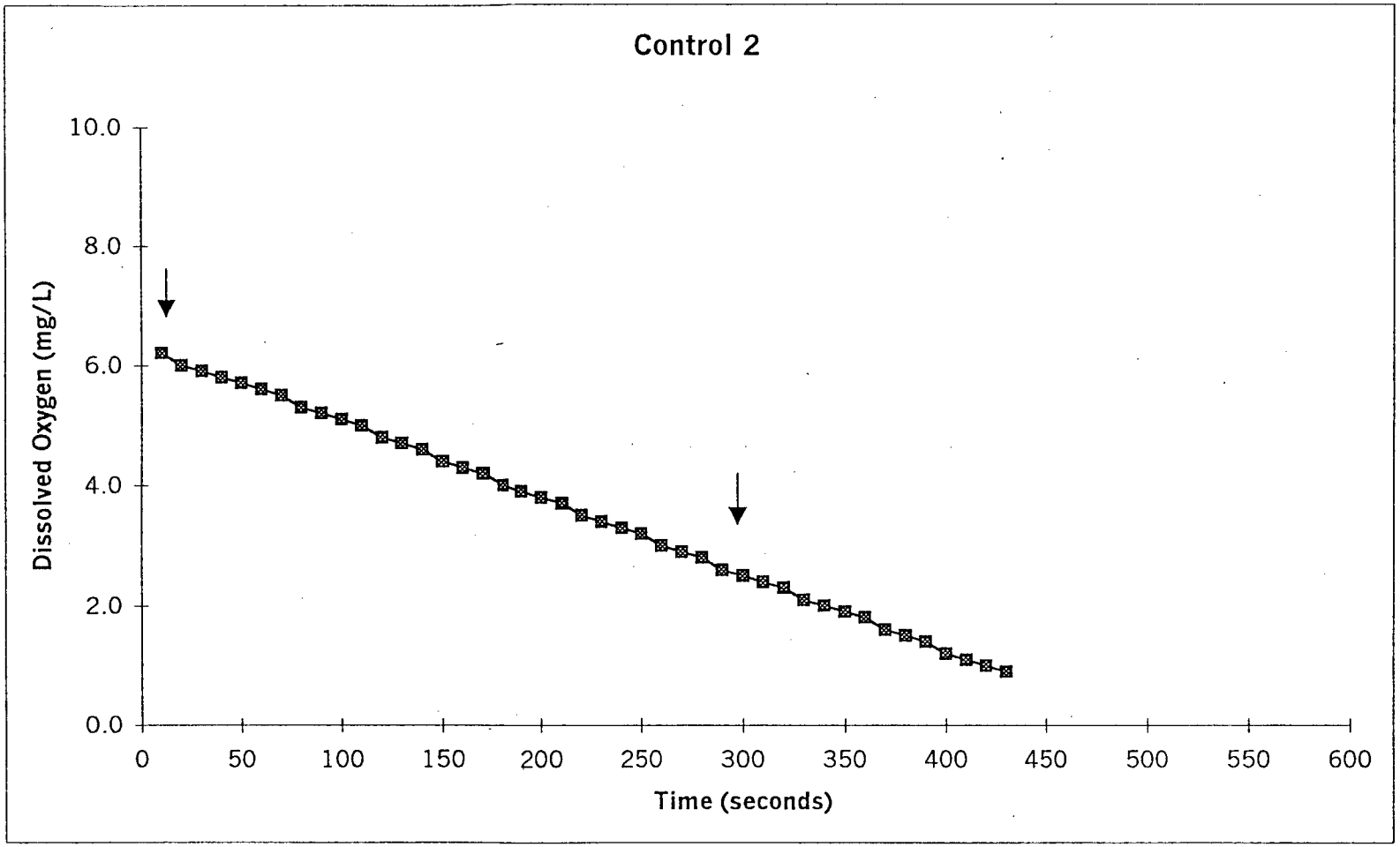

Figure 2. Dissolved Oxygen Concentration Over Time, Control 1. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

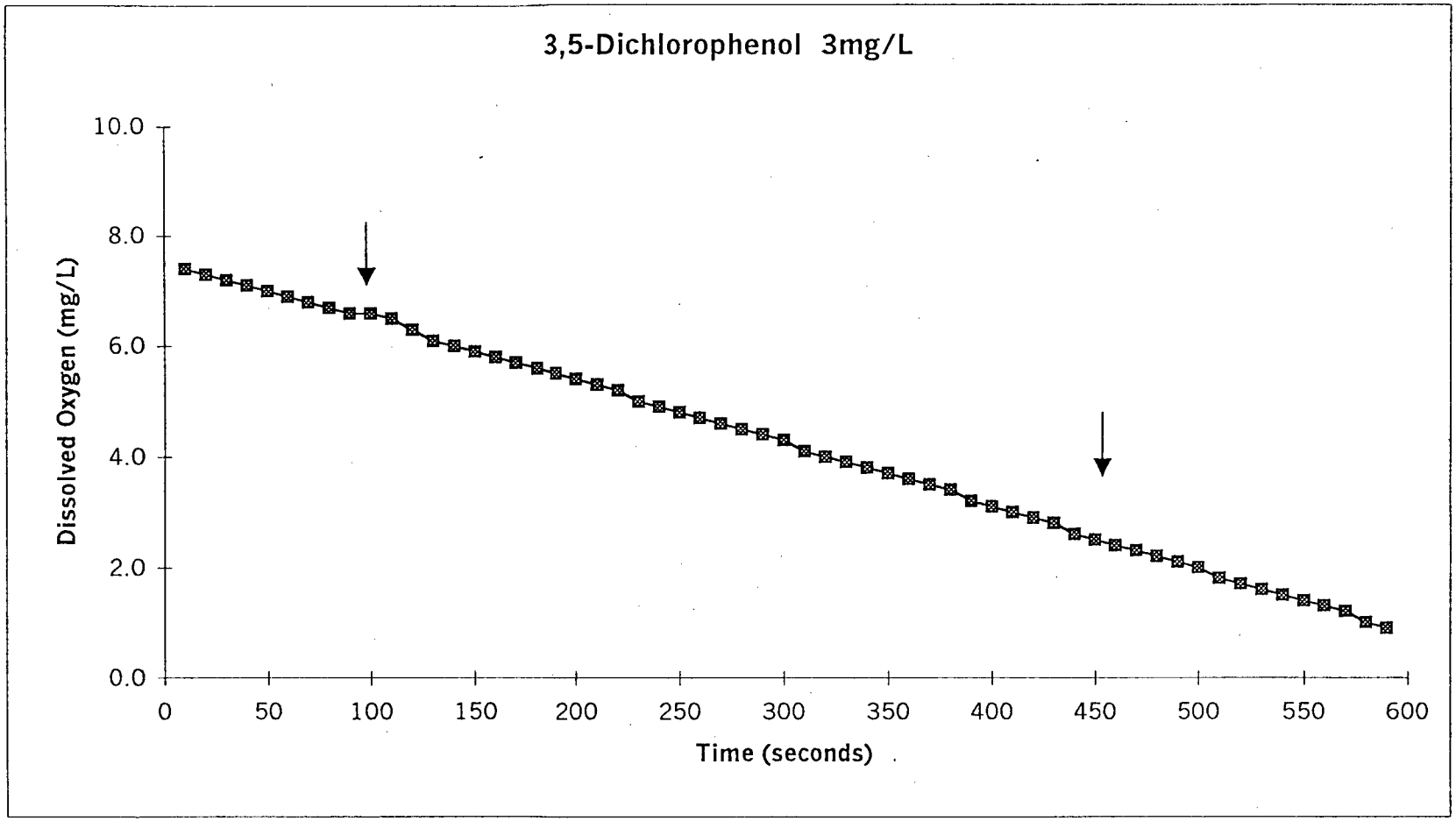

Figure 3. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 3 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

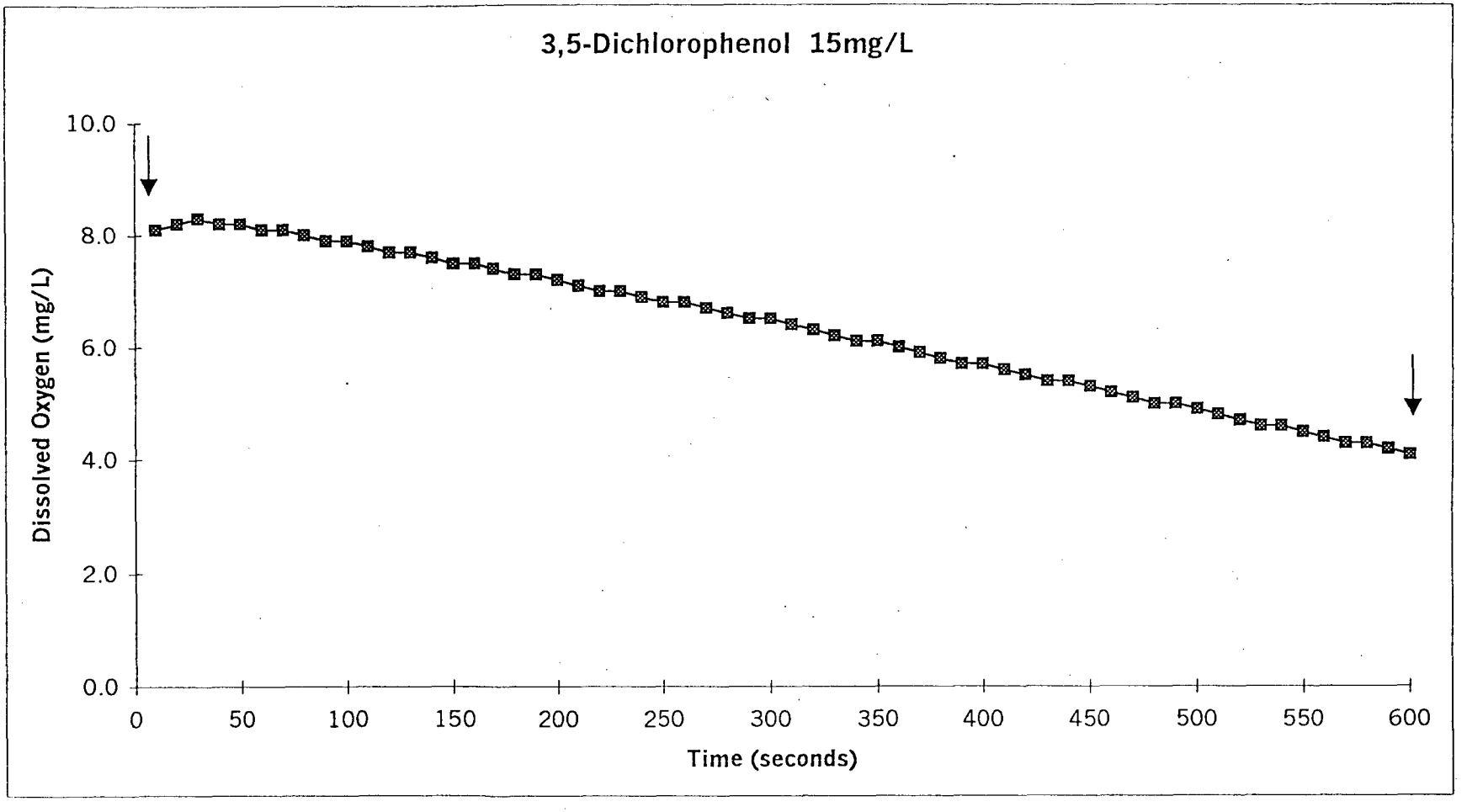

Figure 4. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 15 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

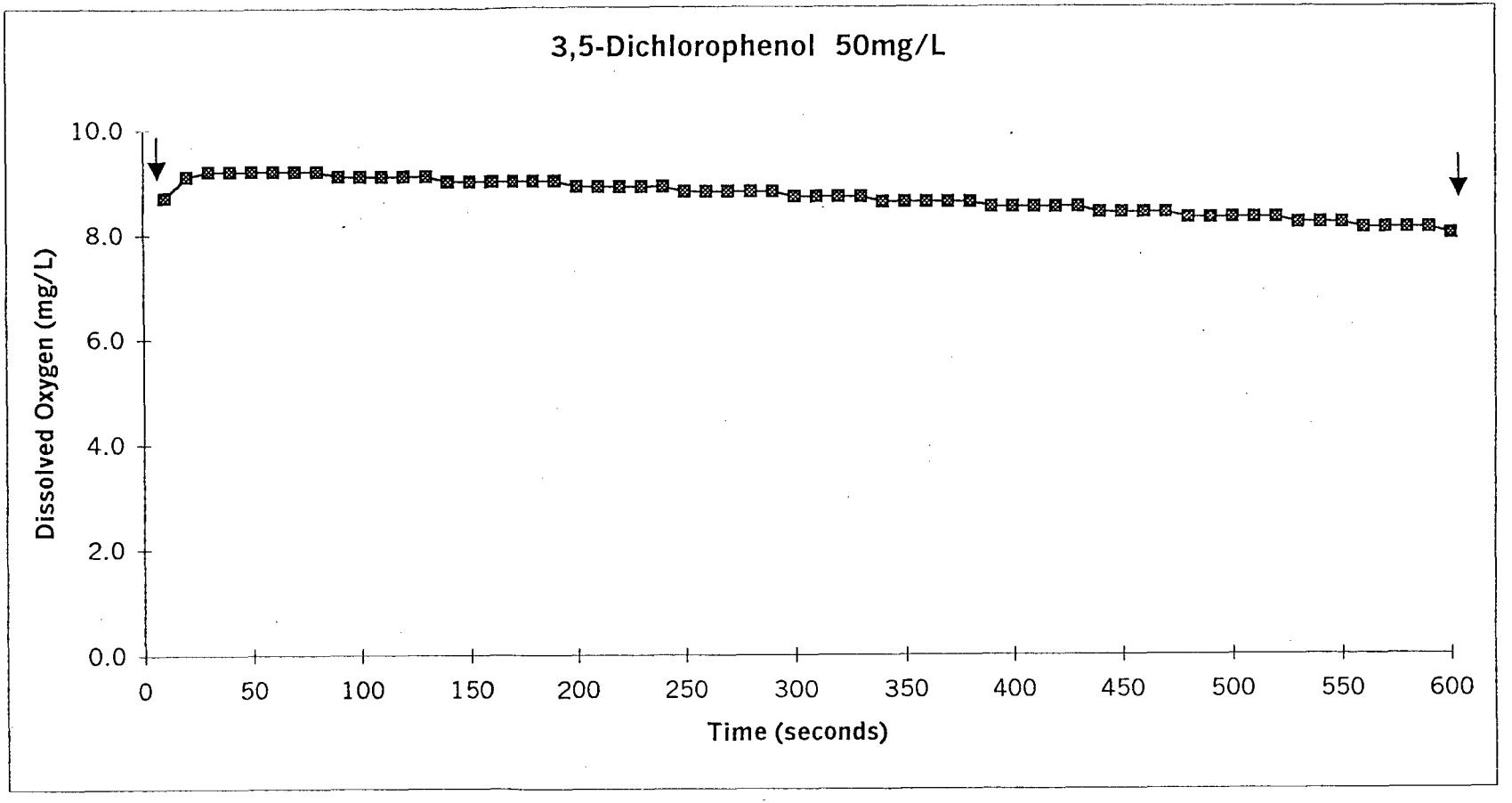

Figure 5. Dissolved Oxygen Concentration Over Time, 3,5-Dichlorophenol, 50 mg/L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

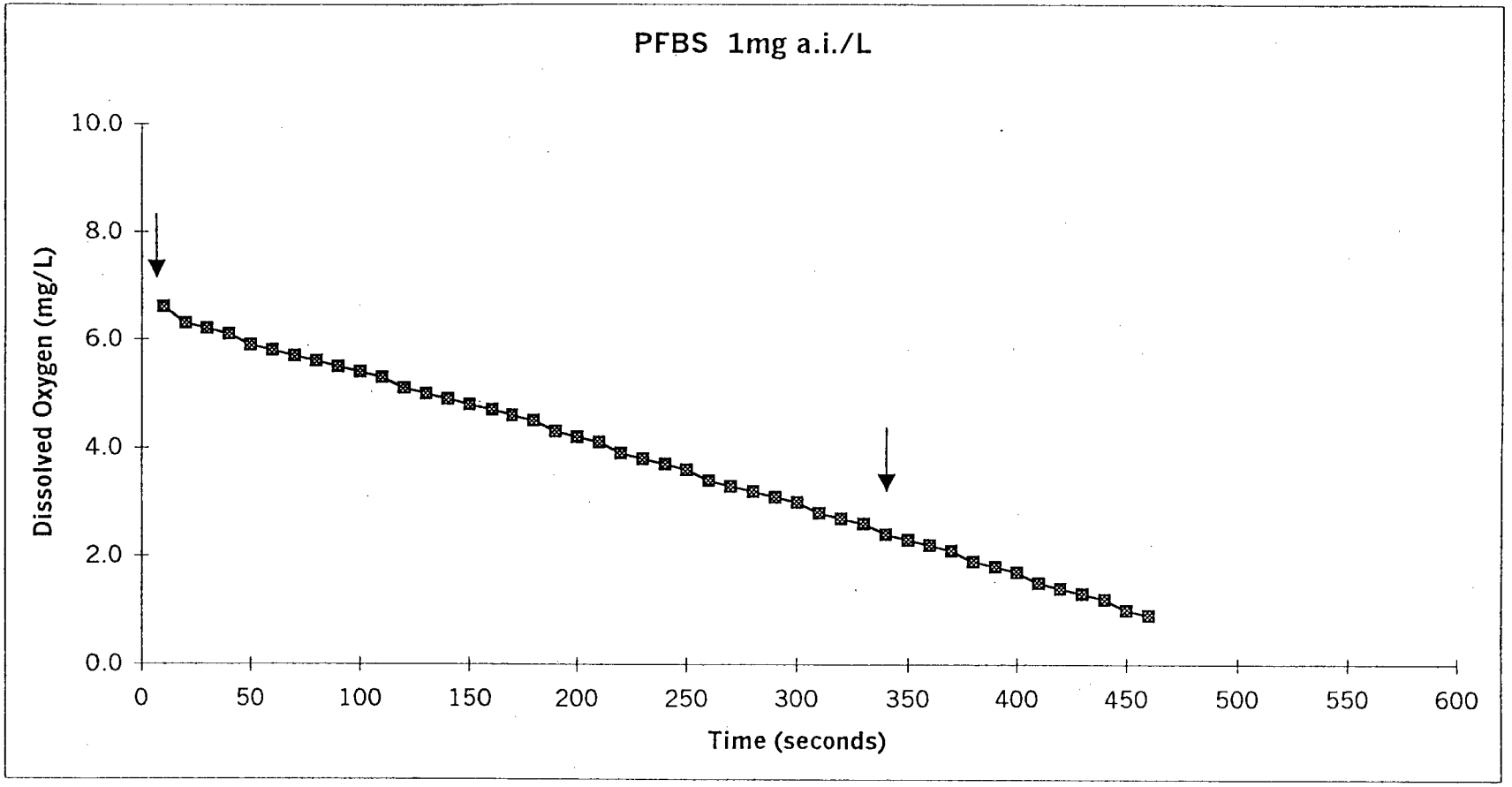

Figure 6. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 1.0 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

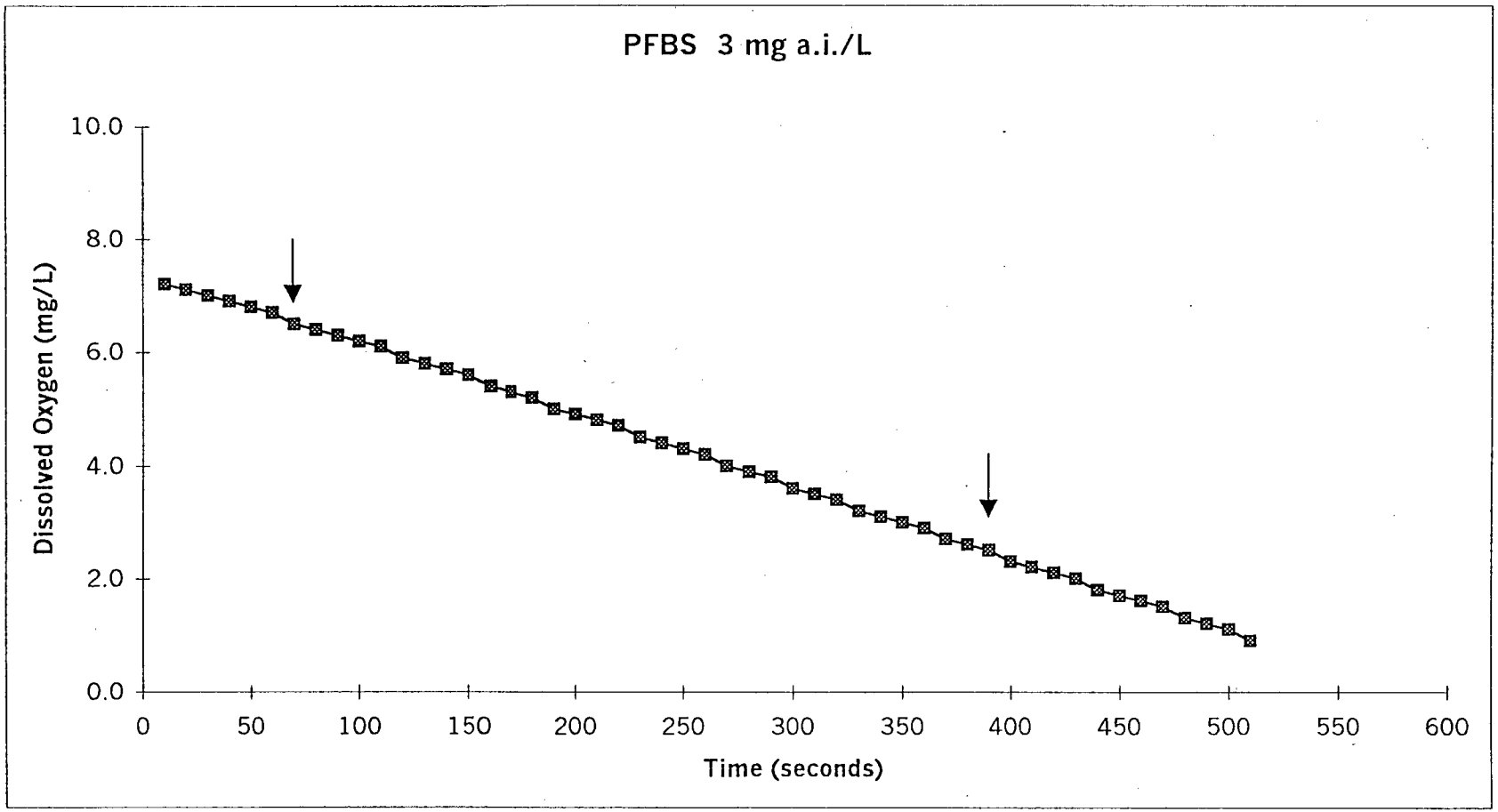

Figure 7. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 3.0 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

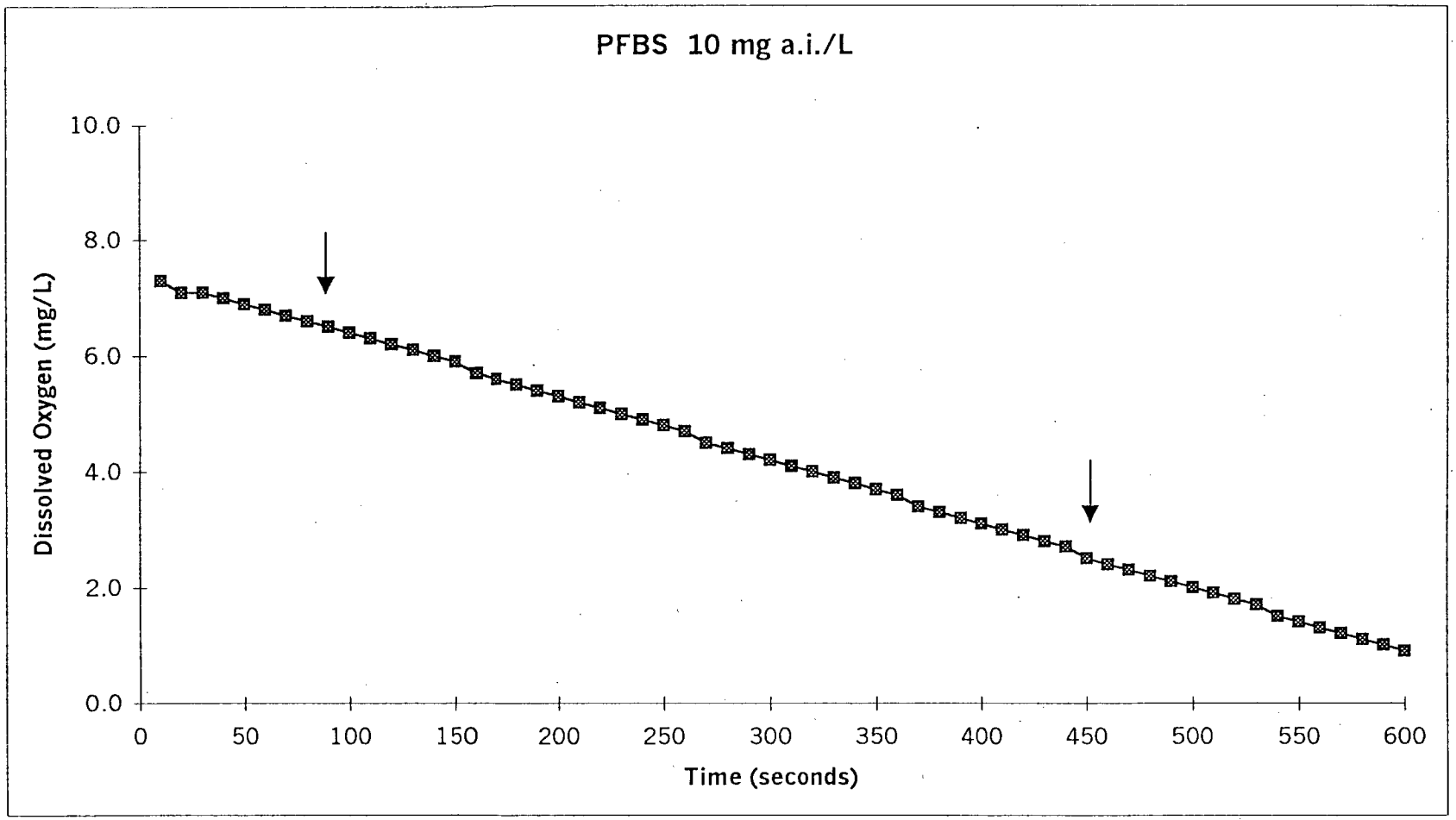

Figure 8. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 10 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

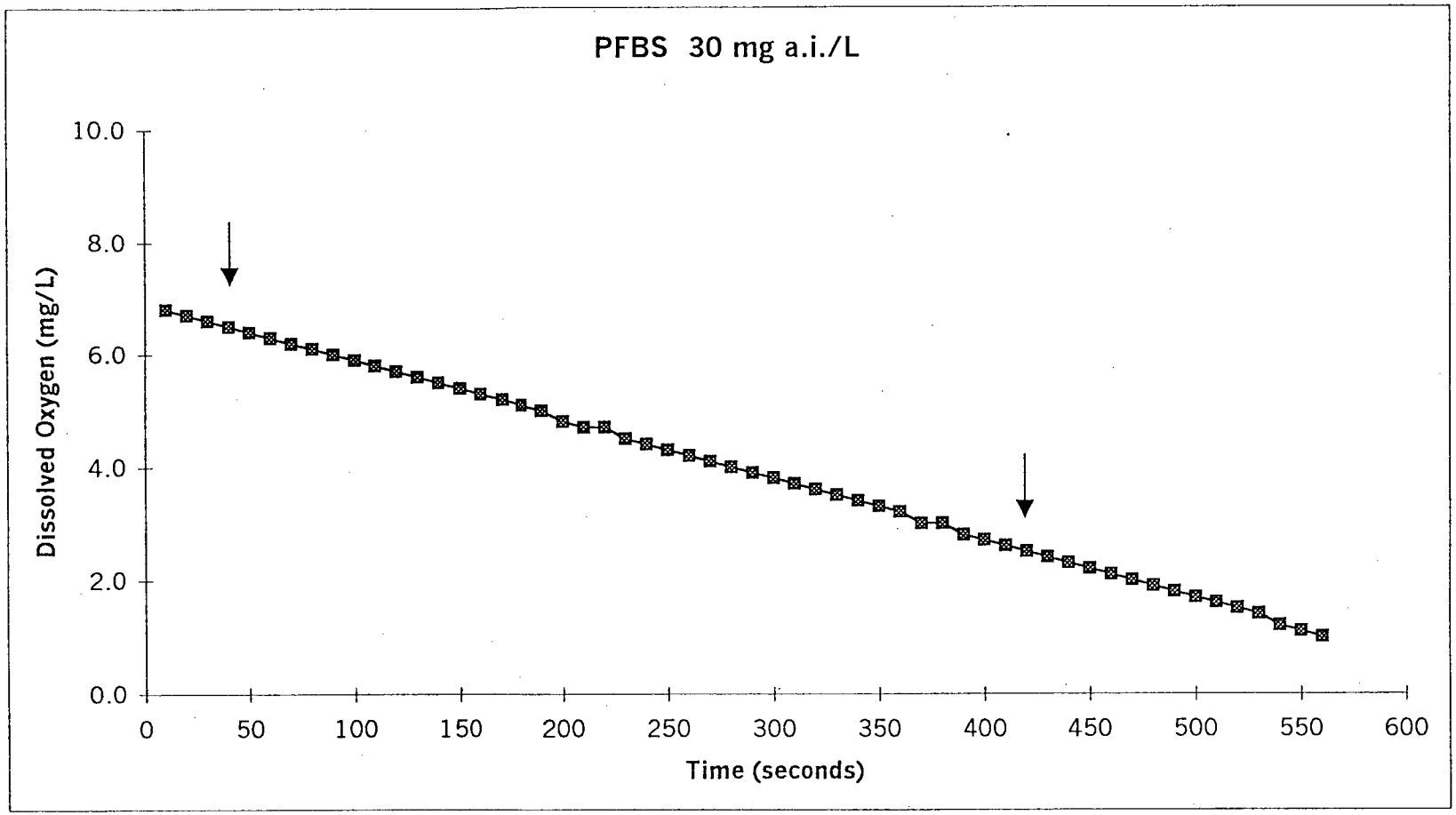

Figure 9. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 30 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

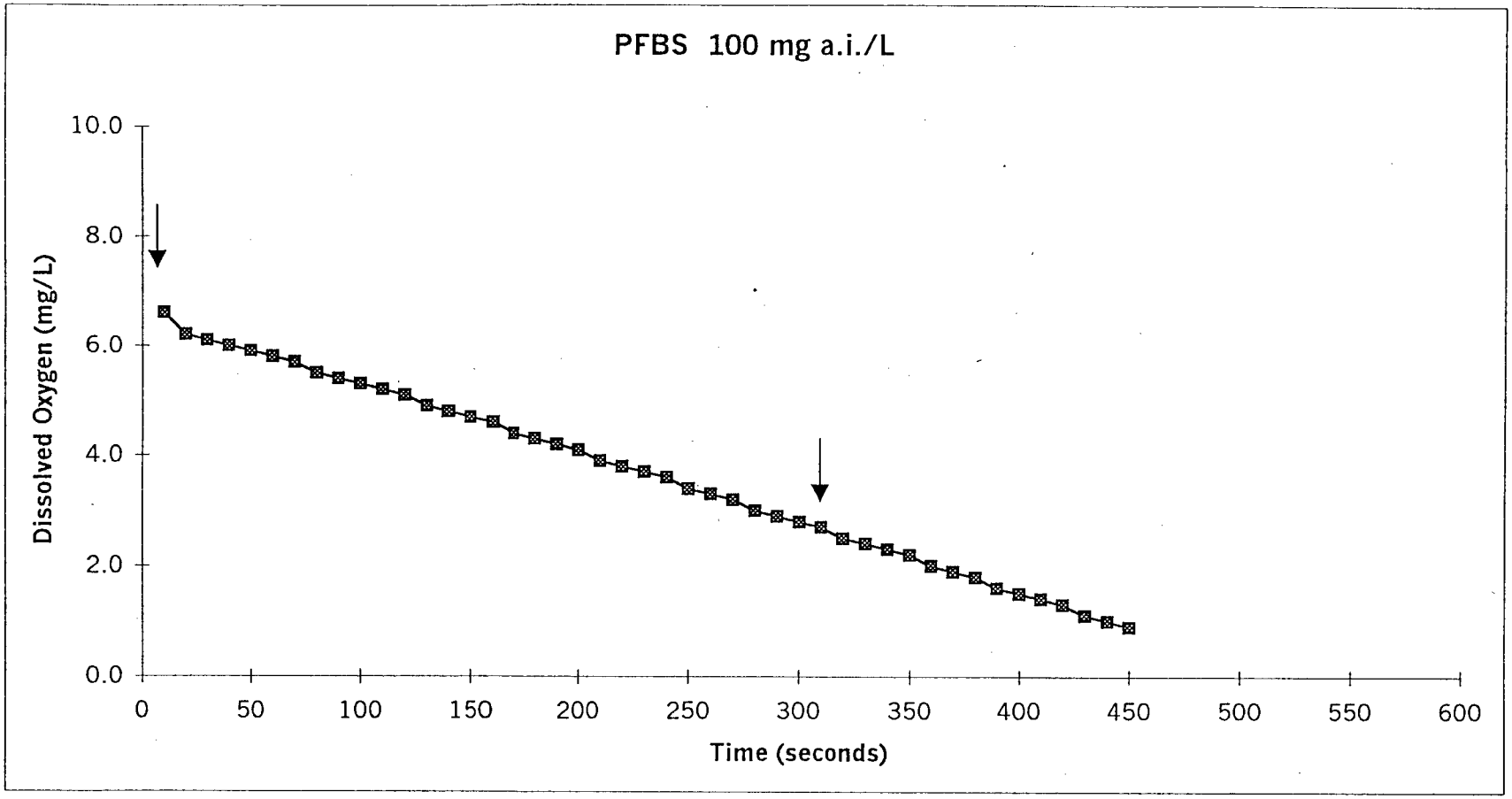

Figure 10. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 100 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

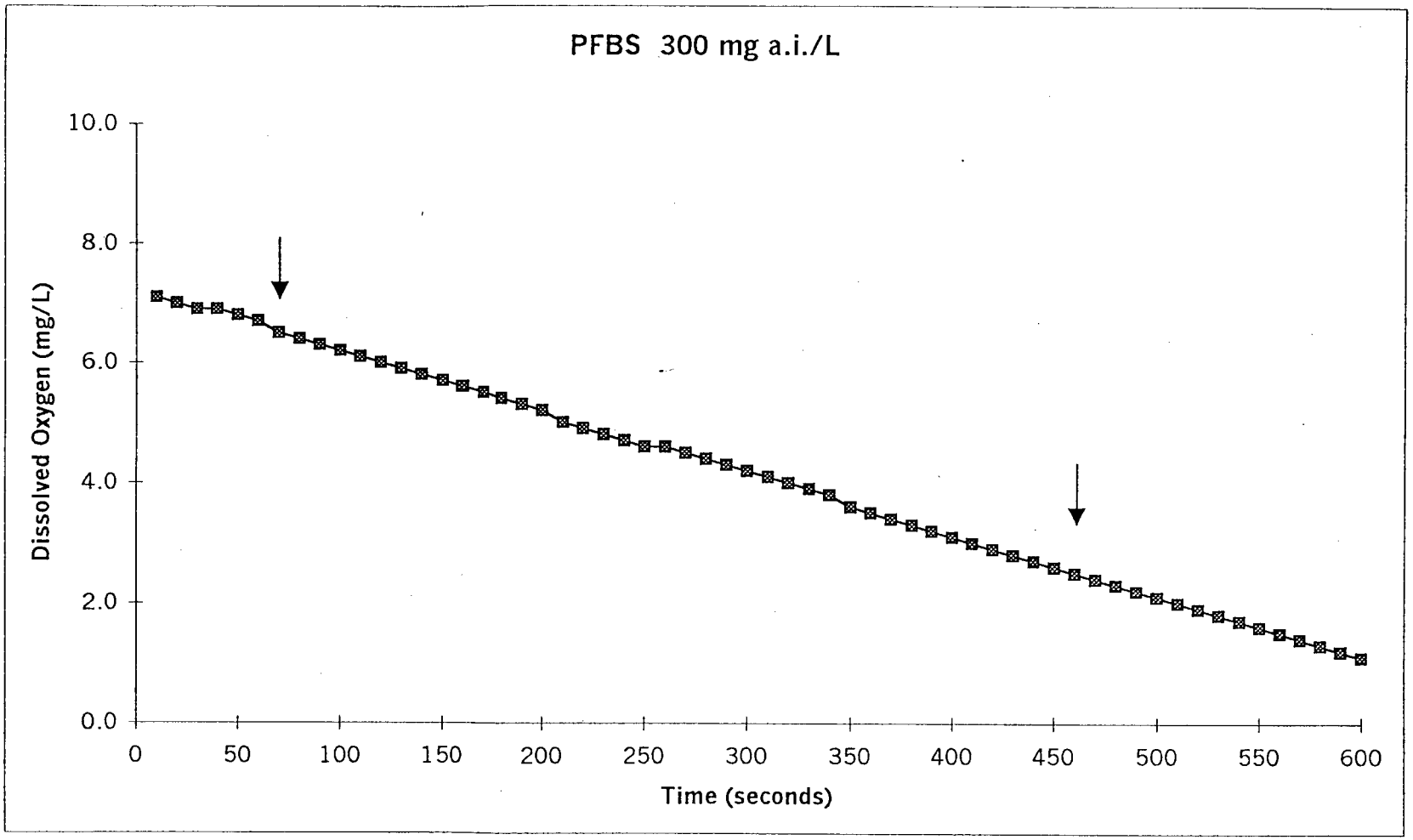

Figure 11. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 300 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

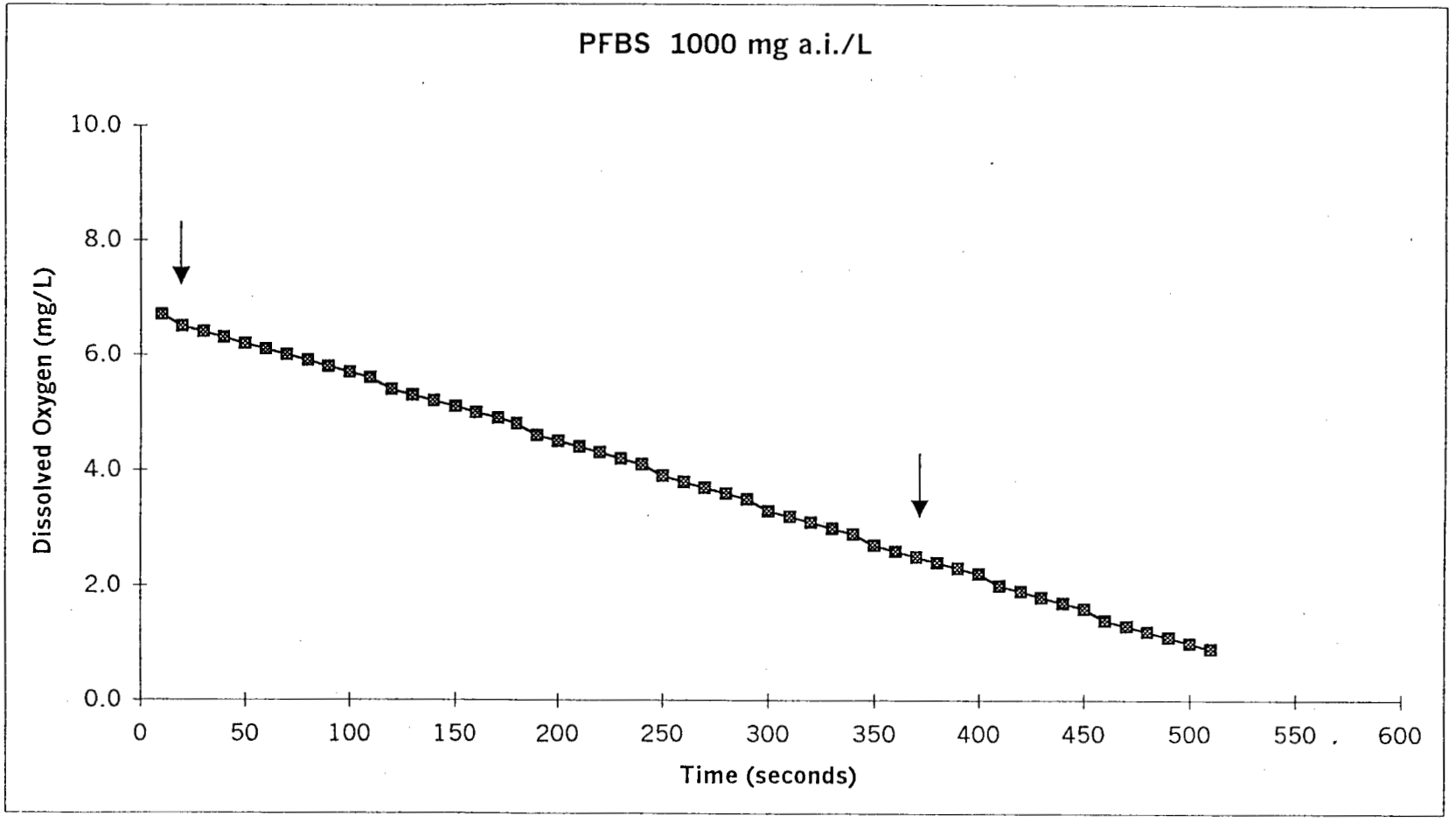

Figure 12. Dissolved Oxygen Concentration Over Time, Perfluorobutanesulfonate (PFBS), 1000 mg a.i./L. Arrows indicate measured dissolved oxygen concentrations used to calculate respiration rate

APPENDIX I

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

Time (min./sec.)	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	PFBS 1 mg a.i./L	PFBS 3 mg a.i./L
00:10	7.5	7.4	**8.1**	**8.7**	**6.6**	7.2
00:20	7.5	7.3	8.2	9.1	6.3	7.1
00:30	7.4	7.2	8.3	9.2	6.2	7.0
00:40	7.3	7.1	8.2	9.2	6.1	6.9
00:50	7.1	7.0	8.2	9.2	5.9	6.8
01:00	7.0	6.9	8.1	9.2	5.8	6.7
01:10	6.9	6.8	8.1	9.2	5.7	**6.5**
01.20	6.8	6.7	8.0	9.2	5.6	6.4
01.30	6.7	6.6	7.9	9.1	5.5	6.3
01.40	6.6	6.6	7.9	9.1	5.4	6.2
01:50	6.5	**6.5**	7.8	9.1	5.3	6.1
02:00	**6.5**	6.3	7.7	9.1	5.1	5.9
02:10	6.4	6.1	7.7	9.1	5.0	5.8
02:20	6.2	6.0	7.6	9.0	4.9	5.7
02:30	6.1	5.9	7.5	9.0	4.8	5.6
02:40	6.0	5.8	7.5	9.0	4.7	5.4
02:50	5.9	5.7	7.4	9.0	4.6	5.3
03:00	5.8	5.6	7.3	9.0	4.5	5.2
03:10	5.7	5.5	7.3	9.0	4.3	5.0
03:20	5.6	5.4	7.2	8.9	4.2	4.9
03:30	5.4	5.3	7.1	8.9	4.1	4.8
03:40	5.3	5.2	7.0	8.9	3.9	4.7
03:50	5.2	5.0	7.0	8.9	3.8	4.5
04:00	5.1	4.9	6.9	8.9	3.7	4.4
04:10	4.9	4.8	6.8	8.8	3.6	4.3
04:20	4.8	4.7	6.8	8.8	3.4	4.2
04:30	4.7	4.6	6.7	8.8	3.3	4.0
04:40	4.6	4.5	6.6	8.8	3.2	3.9
04:50	4.5	4.4	6.5	8.8	3.1	3.8
05:00	4.3	4.3	6.5	8.7	3.0	3.6
05:10	4.2	4.1	6.4	8.7	2.8	3.5
05:20	4.0	4.0	6.3	8.7	2.7	3.4
05:30	3.9	3.9	6.2	8.7	2.6	3.2
05:40	3.8	3.8	6.1	8.6	**2.4**	3.1
05:50	3.7	3.7	6.1	8.6	2.3	3.0
06:00	3.5	3.6	6.0	8.6	2.2	2.9
06:10	3.4	3.5	5.9	8.6	2.1	2.7
06:20	3.3	3.4	5.8	8.6	1.9	2.6
06:30	3.2	3.2	5.7	8.5	1.8	**2.5**
06:40	3.1	3.1	5.7	8.5	1.7	2.3
06:50	2.9	3.0	5.6	8.5	1.5	2.2
07:00	2.8	2.9	5.5	8.5	1.4	2.1
07.10	2.7	2.8	5.4	8.5	1.3	2.0
07.20	2.6	2.6	5.4	8.4	1.2	1.8
07.30	**2.5**	**2.5**	5.3	8.4	1.0	1.7
07:40	2.4	2.4	5.2	8.4	0.9	1.6
07:50	2.2	2.3	5.1	8.4		1.5
08:00	2.1	2.2	5.0	8.3		1.3
08:10	2.0	2.1	5.0	8.3		1.2
08:20	1.9	2.0	4.9	8.3		1.1
08:30	1.7	1.8	4.8	8.3		0.9
08:40	1.6	1.7	4.7	8.3		
08:50	1.5	1.6	4.6	8.2		
09:00	1.4	1.5	4.6	8.2		
09:10	1.3	1.4	4.5	8.2		
09:20	1.1	1.3	4.4	8.1		
09:30	1.0	1.2	4.3	8.1		
09:40	0.9	1.0	4.3	8.1		
09:50		0.9	4.2	8.1		
10:00			**4.1**	**8.0**		

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

APPENDIX I (Continued)

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

Time (min.)	Treatment Dissolved Oxygen (mg/L)					
	PFBS 10 mg a.i./L	PFBS 30 mg a.i./L	PFBS 100 mg a.i./L	PFBS 300 mg a.i./L	PFBS 1000 mg a.i./L	Control 2
00:10	7.3	6.8	**6.6**	7.1	6.7	**6.2**
00:20	7.1	6.7	6.2	7.0	**6.5**	6.0
00:30	7.1	6.6	6.1	6.9	6.4	5.9
00:40	7.0	**6.5**	6.0	6.9	6.3	5.8
00:50	6.9	6.4	5.9	6.8	6.2	5.7
01:00	6.8	6.3	5.8	6.7	6.1	5.6
01:10	6.7	6.2	5.7	**6.5**	6.0	5.5
01.20	6.6	6.1	5.5	6.4	5.9	5.3
01.30	**6.5**	6.0	5.4	6.3	5.8	5.2
01.40	6.4	5.9	5.3	6.2	5.7	5.1
01:50	6.3	5.8	5.2	6.1	5.6	5.0
02:00	6.2	5.7	5.1	6.0	5.4	4.8
02:10	6.1	5.6	4.9	5.9	5.3	4.7
02:20	6.0	5.5	4.8	5.8	5.2	4.6
02:30	5.9	5.4	4.7	5.7	5.1	4.4
02:40	5.7	5.3	4.6	5.6	5.0	4.3
02:50	5.6	5.2	4.4	5.5	4.9	4.2
03:00	5.5	5.1	4.3	5.4	4.8	4.0
03:10	5.4	5.0	4.2	5.3	4.6	3.9
03:20	5.3	4.8	4.1	5.2	4.5	3.8
03:30	5.2	4.7	3.9	5.0	4.4	3.7
03:40	5.1	4.7	3.8	4.9	4.3	3.5
03:50	5.0	4.5	3.7	4.8	4.2	3.4
04:00	4.9	4.4	3.6	4.7	4.1	3.3
04:10	4.8	4.3	3.4	4.6	3.9	3.2
04:20	4.7	4.2	3.3	4.6	3.8	3.0
04:30	4.5	4.1	3.2	4.5	3.7	2.9
04:40	4.4	4.0	3.0	4.4	3.6	2.8
04:50	4.3	3.9	2.9	4.3	3.5	2.6
05:00	4.2	3.8	2.8	4.2	3.3	**2.5**
05:10	4.1	3.7	2.7	4.1	3.2	2.4
05:20	4.0	3.6	**2.5**	4.0	3.1	2.3
05:30	3.9	3.5	2.4	3.9	3.0	2.1
05:40	3.8	3.4	2.3	3.8	2.9	2.0
05:50	3.7	3.3	2.2	3.6	2.7	1.9
06:00	3.6	3.2	2.0	3.5	2.6	1.8
06:10	3.4	3.0	1.9	3.4	**2.5**	1.6
06:20	3.3	3.0	1.8	3.3	2.4	1.5
06:30	3.2	2.8	1.6	3.2	2.3	1.4
06:40	3.1	2.7	1.5	3.1	2.2	1.2
06:50	3.0	2.6	1.4	3.0	2.0	1.1
07:00	2.9	**2.5**	1.3	2.9	1.9	1.0
07.10	2.8	2.4	1.1	2.8	1.8	0.9
07.20	2.7	2.3	1.0	2.7	1.7	
07.30	**2.5**	2.2	0.9	2.6	1.6	
07:40	2.4	2.1		**2.5**	1.4	
07:50	2.3	2.0		2.4	1.3	
08:00	2.2	1.9		2.3	1.2	
08:10	2.1	1.8		2.2	1.1	
08:20	2.0	1.7		2.1	1.0	
08:30	1.9	1.6		2.0	0.9	
08:40	1.8	1.5		1.9		
08:50	1.7	1.4		1.8		
09:00	1.5	1.2		1.7		
09:10	1.4	1.1		1.6		
09:20	1.3	1.0		1.5		
09:30	1.2	0.9		1.4		
09:40	1.1			1.3		
09:50	1.0			1.2		
10:00	0.9			1.1		

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

APPENDIX II

Graphical Presentation of Percent Inhibition and PFBS Concentration

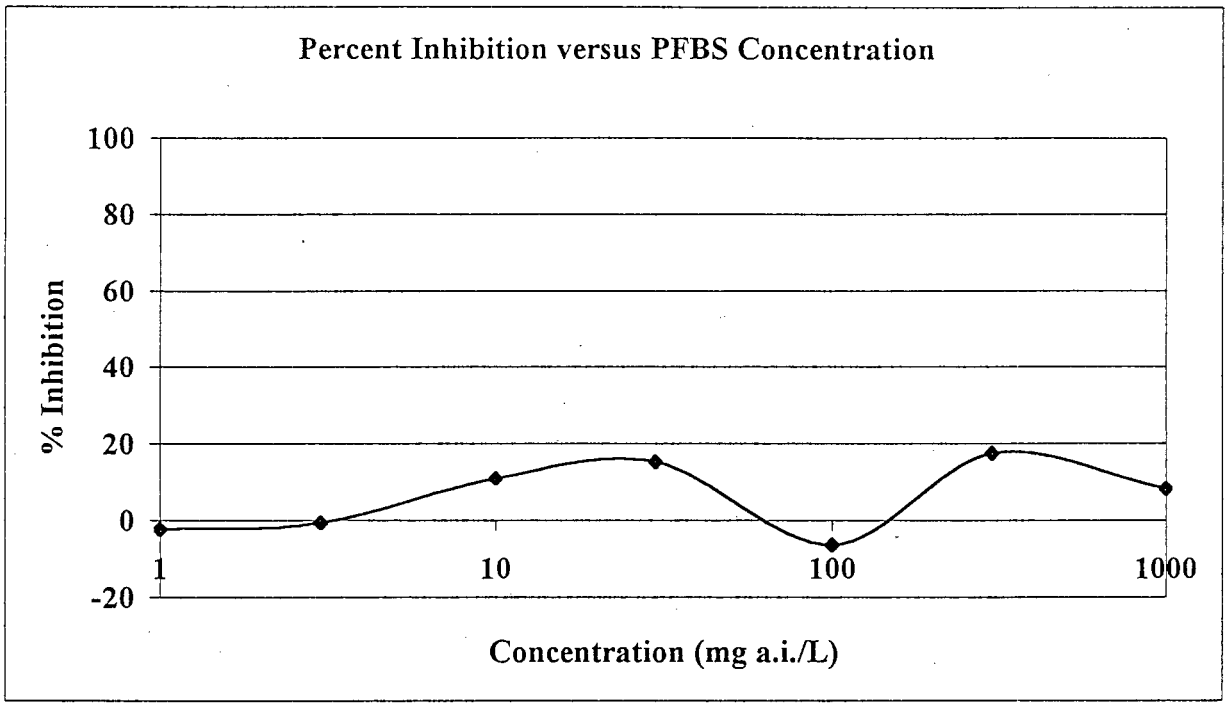

Figure 1. Percent Inhibition versus PFBS Concentration

APPENDIX III

Protocol and Protocol Amendments

PROTOCOL

PFBS: 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 Environmental Lab Project No. E00-1429

Submitted to

3M Corporation
Environmental Laboratory
935 Bush Avenue
St. Paul, Minnesota 55144

Wildlife International, Ltd.

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

October 19, 2000

SANITIZED

Wildlife International, Ltd.

PFBS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

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

SPONSOR'S REPRESENTATIVE:

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: 11/20/2000 Experimental Termination Date: 11/30/2000

Project No.: 454E-102

Test Concentrations: 1, 3, 10, 30, 100, 300, 1000 mg/L

Test Substance No.: 5292 Reference Substance No. (if applicable): 579

PROTOCOL APPROVAL

Edward C. Schaefer
STUDY DIRECTOR — DATE 11/17/2000

LABORATORY MANAGEMENT — DATE 11/17/00

DATE 10/30/00

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 Perfluorobutanesulfonate, potassium salt (hereafter referred to as PFBS) 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

The test substance will be PFBS. 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 initiation of 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 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 Nanopure® 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 I) 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 Nanopure® 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 Nanopure® 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:

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

where

R_t = 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.

Wildlife International, Ltd.

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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 (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. 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.

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APPENDIX I. 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 Nanopure® 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: PFBS: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

PROTOCOL NO.: 454/101900/209aSUB454 AMENDMENT NO.: 1

SPONSOR: 3M Corporation PROJECT NO.: 454E-102

EFFECTIVE DATE: November 27, 2000

AMENDMENT: Test Inoculum, page -4-

DELETE: 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.

ADD: Activated sludge from the Denton Wastewater Treatment Plant will be used as the inoculum for the test. The Denton Facility receives wastes from predominantly domestic sources.

REASON: Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland has been closed.

P.C. Lloyd Jr. 11/27/00
STUDY DIRECTOR DATE

LABORATORY MANAGEMENT 11/28/00
DATE

12/7/00
DATE

QA KH 11-28-00

AMENDMENT TO STUDY PROTOCOL

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

PROTOCOL NO.: 454/101900/209aSUB454 **AMENDMENT NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454E-102

EFFECTIVE DATE: November 27, 2000

AMENDMENT: Test Inoculum, page -4-

DELETE: 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.

ADD: Activated sludge from the Denton Wastewater Treatment Plant will be used as the inoculum for the test. The Denton Facility receives wastes from predominantly domestic sources.

REASON: Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland has been closed.

STUDY DIRECTOR — DATE 11/27/00

LABORATORY MANAGEMENT — DATE 11/28/00

DATE 12/7/00

QA KH 11-28-00

DEC 09 2003

SANITIZED

AMENDMENT TO STUDY PROTOCOL

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

PROTOCOL NO.: 454/101900/209aSUB454 AMENDMENT NO.: 2

SPONSOR: 3M Corporation PROJECT NO.: 454E-102

EFFECTIVE DATE: November 27, 2000

AMENDMENT: Stock Solution Preparation, page -4-

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®).

ADD: Test substance stock solutions will be prepared at nominal concentrations of 1000 and 2000 mg active/L in high quality water (e.g. Nanopure®).

REASON: A more concentrated stock solution is needed to dose the 1000 mg active/L treatment.

E.C. [illegible]
STUDY DIRECTOR

11/27/00
DATE

[illegible]
LABORATORY MANAGEMENT

11/28/00
DATE

12/7/00
DATE

QA
KH 11-28-00

SANITIZED

AMENDMENT TO STUDY PROTOCOL

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

PROTOCOL NO.: 454/101900/209aSUB454 AMENDMENT NO.: 3

SPONSOR: 3M Corporation PROJECT NO.: 454E-102

EFFECTIVE DATE: January 09, 2001

AMENDMENT: Test Concentrations, page -2-

DELETE: Test Concentration: 1, 3, 10, 30, 100, 300, 1000 mg/L

ADD: Test Concentration: 1, 3, 10, 30, 100, 300, 1000 mg A/L

REASON: Entry error by Study Director

AMENDMENT: Calculations, page --

ADD: The respiration rate will calculated using the following equation:

Respiration Rate = (initial DO – final DO)/(final time – initial time)

REASON: Protocol did not contain the equation used to calculate the respiration rate

Edward C. Bell, Jr. 1/9/01
STUDY DIRECTOR DATE

LABORATORY MANAGEMENT 2/27/01
DATE

2/5/01
DATE

QA KH 1-19-01