TETRABROMOBISPHENOL-A: AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

WILDLIFE INTERNATIONAL, LTD PROJECT NUMBER: 439E-107A

Organisation for Economic Cooperation and Development
OECD Guideline 209

and

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

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STUDY INITIATION DATE: November 21, 2001

STUDY COMPLETION DATE: March 27, 2002

SUBMITTED TO:

American Chemistry Council's
Brominated Flame Retardant Industry Panel
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Tetrabromobisphenol-A: An Activated Sludge, Respiration Inhibition Test

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439E-107A

STUDY COMPLETION: March 27, 2002

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.

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

The homogeneity and stability of the reference material in the carrier was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Edward C. Schaefer
Manager, Biodegradation

27 MARCH 2002
DATE

SPONSOR REPRESENTATIVE:

Ms. Wendy Sherman
American Chemistry Council's
Brominated Flame Retardant Industry Panel

March 25, 2002
DATE

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: 439E-107			
Test Substance Preparation And Test Initiation	December 12, 2001	December 12, 2001	March 22, 2002
D. O. Measurements	December 12, 2001	December 12, 2001	December 14, 2001
Definitive Trial: 439E-107A			
Test Substance Preparation	December 19, 2001	December 19, 2001	March 26, 2002
Data & Draft Report	January 14 &18, 2002	January 18, 2002	January 22, 2002
Final Report	March 26, 2002	March 26, 2002	March 27, 2002

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

March 27, 2002
DATE

REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Tetrabromobisphenol-A: An Activated Sludge, Respiration Inhibition Test

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439E-107A

STUDY DIRECTOR:

Edward C. Schaefer
Manager, Biodegradation

27 MARCH 2002
DATE

MANAGEMENT:

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

27 Mar 02
DATE

STUDY INFORMATION

Study Initiation Date:	November 21, 2001
Experimental Start Date:	December 11, 2001
Experimental Termination Date:	December 19, 2001
Study Completion Date:	March 27, 2002

Study Director:	Edward C. Schaefer
Sponsor:	American Chemistry Council's Brominated Flame Retardant Industry Panel 1300 Wilson Boulevard Arlington, Virginia 22209
Sponsor's Representative:	Ms. Wendy Sherman
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

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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 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 nominal concentrations of 3, 15 and 50 mg/L. The test substance was dosed at a limit concentration of 15 mg/L. After an exposure period of approximately three hours, the respiration rates of the test solutions were measured using a dissolved oxygen meter. The individual respiration rates of the two controls were 15.3 and 17.1 mg O_2/L/hr. The difference between the two control respiration rates was 10.5% and 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 9.6 mg/L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test. An average of approximately 1.9 percent inhibition was observed in the treatment group. Following is a summary of the results:

Treatment/Nominal Concentration	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	15.3	NA
Control 2	17.1	NA
3,5-dichlorophenol 3 mg/L	15.3	5.9
3,5-dichlorophenol 15 mg/L	4.9	69.9
3,5-dichlorophenol 50 mg/L	2.4	85.2
Tetrabromobisphenol-A 15 mg/L	15.9	1.9
Tetrabromobisphenol-A 15 mg/L	15.9	1.9
Tetrabromobisphenol-A 15 mg/L	15.9	1.9

NA – Not applicable

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 the American Chemistry Council's Brominated Flame Retardant Industry Panel 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 439E-107A in the archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to assess the effects of tetrabromobisphenol-A oxide 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 nominal concentrations of 3, 15 and 50 mg/L. The test substance was tested at a limit concentration of 15 mg/L, in triplicate.

MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, "Tetrabromobisphenol A: An Activated Sludge, Respiration Inhibition Test," (Appendix II). 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 used in this study was a composite of the following three samples:

Manufacturer:	Bromide Compounds Ltd
Sample ID:	Tetrabromobisphenol-A
Description	Powder
Purity	>99% Tetrabromobisphenol-A
Lot No.:	000135
CAS No:	Not given
Expiration Date:	Not given
Date Received:	August 17, 2000
Wildlife International, Ltd. ID:	5354

Manufacturer:	Great Lakes Chemical Corporation
Sample ID:	Tetrabromobisphenol A
Description	White powder
Purity	Not given
Batch No :	008JG21C
CAS No:	00079-94-7
Expiration Date:	Not given
Date Received:	July 25, 2000
Wildlife International, Ltd. ID:	5315

Manufacturer:	Albemarle Corporation
Sample ID:	Tetrabromobisphenol-A (TBBPA)
Description	White powder
Purity	Not given
Lot No.:	25938C-1
CAS No.	79-94-7
Expiration Date:	Not given
Date Received:	July 27, 2000
Wildlife International, Ltd. ID:	5318

The composite tetrabromobisphenol-A sample was prepared on September 19, 2000 and was assigned Wildlife International Ltd. identification number 5381. The composite sample was prepared by combining equal parts of the three manufacturers' products and mixing for approximately ten minutes.

The test substance was administered to the treatment group by direct weight 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 December 18, 2001. The Denton facility receives wastes from predominately domestic sources. The sludge was sieved using a 2 mm screen and allowed to settle for approximately 30 minutes. After the settling period, the supernatant was removed and the total suspended solids (TSS) concentration of the settled sludge was determined.

The sludge was maintained in the laboratory for 1 day prior to use. Approximately 50 mL of synthetic sewage (Protocol, Appendix II) 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 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 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:

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

Percent inhibition was calculated using the following equation:

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

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 (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). The EC50 value for the reference group was calculated using nonlinear interpolation.

RESULTS AND DISCUSSION

The temperature range during the maintenance of the sludge and during the test was 20-22° C. The measured total suspended solids (TSS) concentration and pH of the sludge on the day of testing was 3640 mg/L and 7.8, respectively.

Respiration rates and percent inhibitions are presented in Table 1. The respiration rates in the two controls were 15.3 and 17.1 mg O_2/L/hr. The difference between the two control respiration rates was 10.5% and 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 9.6 mg/L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test.

Minimal inhibitory effects upon respiration were observed at a tetrabromobisphenol-A concentration of 15 mg/L. The average respiration rate for the treatment group was 15.9 ± 0.0 O_2/L/hr and was slightly lower than the average respiration rate of the control (16.2 ± 1.3 mg O_2/L/hr). The average percent inhibition observed was approximately 1.9%.

CONCLUSION

Minimal inhibitory effects upon respiration were observed at a tetrabromobisphenol-A concentration of 15 mg/L. The average percent inhibition observed was approximately 1.9%.

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.

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/Nominal Concentration	Respiration Rate mg O_2/L/hour	Percent Inhibition
Control 1	15.3	NA
Control 2	17.1	NA
3,5-dichlorophenol 3 mg/L	15.3	5.9
3,5-dichlorophenol 15 mg/L	4.9	69.9
3,5-dichlorophenol 50 mg/L	2.4	85.2
Tetrabromobisphenol-A 15 mg/L	15.9	1.9
Tetrabromobisphenol-A 15 mg/L	15.9	1.9
Tetrabromobisphenol-A 15 mg/L	15.9	1.9

NA – Not applicable.

APPENDIX I

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

Time (min./sec)	Control 1	Reference			Treatment			Control 2
		3 mg/L	15 mg/L	50 mg/L	Rep A 15 mg/L	Rep B 15 mg/L	Rep C 15 mg/L	
00:10	**8.4**	**8.4**	**8.5**	**9.0**	**8.0**	**8.2**	**8.1**	**8.1**
00.20	8 4	8 4	8.7	9.1	8 0	8 2	8.1	8 1
00:30	8 3	8 3	8 7	9 2	8 0	8 2	8 1	8 0
00 40	8 3	8 3	8 6	9 2	7.9	8 1	8.0	8 0
00:50	8.3	8 2	8 6	9 2	7 9	8 1	8 0	7 9
00.60	8 2	8 2	8.6	9.2	7.9	8 0	7.9	7.9
00 70	8 2	8 2	8.6	9 2	7 8	8 0	7.9	7 9
00 80	8 2	8 1	8.6	9 2	7.8	7.9	7.8	7 8
00 90	8 1	8 1	8 6	9 2	7.7	7 9	7.8	7 8
01 00	8 1	8.1	8.6	9 2	7 7	7 8	7 8	7 7
01 10	8.0	8 0	8 6	9 1	7 6	7.8	7.7	7.7
01 20	8 0	8.0	8.6	9 1	7.6	7.8	7 7	7 6
01:30	8 0	7 9	8 5	9 1	7 6	7 7	7.6	7 6
01 40	7 9	7.9	8.5	9 1	7.5	7.7	7 6	7 5
01 50	7 9	7.9	8 5	9.1	7.5	7.6	7 6	7.5
01:60	7.8	7 8	8 5	9 1	7 4	7.6	7.5	7.4
01 70	7 8	7 8	8 5	9 0	7 4	7.5	7.5	7.4
01.80	7 7	7.7	8 5	9 0	7 3	7 5	7.4	7 4
01:90	7 7	7.7	8 5	9.0	7.3	7.4	7 4	7 3
02:00	7.7	7 6	8.4	9 0	7 2	7.4	7 3	7 3
02.10	7.6	7.6	8.4	9 0	7.2	7.4	7 3	7 2
02 20	7.6	7 6	8 4	9.0	7 2	7 3	7 2	7 2
02 30	7 5	7.5	8.4	9.0	7 1	7 3	7 2	7 1
02 40	7 5	7 5	8 4	9 0	7 1	7.2	7.1	7.1
02:50	7.4	7.4	8.3	9.0	7 0	7.2	7 1	7 0
02:60	7 4	7 4	8.3	9 0	7.0	7.1	7.0	7.0
02 70	7 4	7.4	8.3	9.0	6.9	7 1	7 0	6.9
02 80	7 3	7 3	8.3	9.0	6.9	7 0	7 0	6.9
02.90	7 3	7.3	8.3	9.0	6 8	7.0	6.9	6.8
03 00	7.2	7 2	8.2	8 9	6.8	7 0	6.9	6.8
03.10	7 2	7.2	8.2	8 9	6 8	6 9	6.8	6 7
03 20	7.1	7 2	8 2	8.9	6.7	6.9	6 8	6.7
03:30	7.1	7 1	8 2	8.9	6 7	6 8	6.7	6.6
03 40	7.0	7 1	8 2	8 9	6 6	6.8	6.7	6.6
03:50	7.0	7 0	8.2	8 9	6.6	6.7	6 6	6 5
03.60	6.9	7.0	8 1	8 9	6.6	6 7	6.6	6.5
03 70	6.9	6 9	8 1	8 9	6.5	6 6	6 6	6 4
03:80	6.9	6.9	8.1	8 9	6.5	6 6	6.5	6.4
03 90	6 8	6.8	8 1	8.8	6 4	6 6	6.5	6.3
04 00	6 8	6 8	8 0	8.8	6 4	6 5	6 4	6 3
04 10	6 7	6 8	8 0	8 8	6 3	6.5	6 4	6 2
04 20	6.7	6 7	8.0	8.8	6 3	6.4	6 3	6 2
04 30	6 6	6 7	8.0	8 8	6.2	6.4	6 3	6 1
04 40	6.6	6.6	7.9	8 8	6 2	6.3	6 2	6 1
04.50	6 6	6 6	7 9	8 8	6 1	6.3	6 2	6.1
04:60	6 5	6 5	7 9	8.8	6.1	6.2	6 1	6 0
04.70	6 5	6 5	7.9	8 8	6.1	6.2	6.1	6 0
04:80	6.4	6 4	7 9	8.8	6 0	6 1	6.1	5.9
04 90	6 4	6 4	7 9	8.7	6 0	6 1	6 0	5 9
05:00	6 4	6 4	7 9	8.7	5 9	6 1	6 0	5.8
05.10	6 3	6 3	7.8	8 7	5 9	6.0	5 9	5 8
05 20	6 3	6 3	7 8	8.7	5 8	6 0	5.9	5 7
05 30	6.3	6 2	7 8	8 7	5 8	5.9	5.8	5.7
05:40	6 2	6 2	7 8	8 7	5.7	5 9	5 8	5 6
05 50	6 2	6 1	7 8	8 7	5 7	5 8	5 7	5 6
05:60	6 1	6 1	7 8	8 7	5 6	5 8	5 7	5 5
05 70	6 1	6.1	7 8	8.6	5 6	5 7	5 6	5 5
05.80	6 0	6 0	7 7	8.6	5 5	5 7	5 6	5 4
05 90	6 0	6 0	7.7	8 6	5.5	5 6	5.6	5.4
06 00	**5.9**	**5.9**	**7.7**	**8.6**	**5.4**	**5.6**	**5.5**	**5.3**

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

APPENDIX II

Protocol

PROTOCOL

TETRABROMOBISPHENOL A: AN ACTIVATED SLUDGE,
RESPIRATION INHIBITION TEST

Organization for Economic Cooperation and Development
OECD Guideline 209

and

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

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

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

November 7, 2001

Wildlife International, Ltd.

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TETRABROMOBISPHENOL A: AN ACTIVATED SLUDGE,
RESPIRATION INHIBITION TEST

SPONSOR:	American Chemistry Council's Brominated Flame Retardant Industry Panel 1300 Wilson Boulevard Arlington, Virginia 22209
SPONSOR'S REPRESENTATIVE:	Ms. Wendy Sherman
TESTING FACILITY:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Edward C. Schaefer
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Manager of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 12/04/01 Experimental Termination Date: 12/10/01

Project No.: 439E-107

Test Concentrations: 15 mg/L

Test Substance No.: 5381 Reference Substance No. (if applicable): 5179

PROTOCOL APPROVAL

STUDY DIRECTOR	DATE 11/21/01
LABORATORY MANAGEMENT	DATE 11/21/01
SPONSOR'S REPRESENTATIVE	DATE 11/16/01

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 control, reference, and treatment groups. 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 a limit concentration of 15 mg/l, in triplicate.

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 consisted of a composite of TBBPA samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

Manufacturer	Lot/Batch	Date Received	Wildlife International Ltd. Identification Number
Great Lakes Chemical Corporation	008JG21C	July 25, 2000	5315
Albemarle Corporation	25938C-1	July 27, 2000	5318
Bromine Compounds, Ltd.	000135	August 17, 2000	5354

The composite test substance was assigned Wildlife International Ltd. identification number 5381 and is being stored under ambient conditions.

Wildlife International, Ltd.

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.

The test substance will be administered by direct weight addition. Direct weight addition is the most appropriate route of administration of insoluble materials

Stock Solution Preparation

A stock solution of 3,5-dichlorophenol will be prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with NANO™pure 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 NANO™pure water. The reference substance will be administered by volumetric addition.

Test System

The biological test system is 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 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 conduct dissolved oxygen (DO) measurements. Test mixtures will be identified by project number, test substance identification and test concentration.

Test Inoculum

Activated sludge from the Denton Wastewater Treatment Plant, Denton, Maryland will be used as the inoculum for the test. The sludge will be sieved using a 2 mm screen and then allowed to settle 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 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 amount of test or reference substance, 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 occurs 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 respiration rate will be calculated as follows:

$$Respiration\ Rate = (initial\ DO - final\ DO)/(final\ time - initial\ time)$$

Wildlife International, Ltd.

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

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

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

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.

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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.
16. A copy of the signed protocol and amendments.

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 (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.

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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

IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): Tetrabromobisphenol-A

Reference Standard (if applicable): N/A

Test Substance Sample Code or Batch Number: Wildlife International, Ltd. No. 5381

Test Substance Purity (% Active Ingredient): 98.91% Expiration Date: August 1, 2002

II. Test Substance Characterization

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

III. Test Substance Storage Conditions

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

Ambient temperature; protect from light and moisture

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

Other pertinent stability information:

N/A

IV. Toxicity Information:

Mammalian: Rat LD50 > 5 g/kg Mouse LD50: > 10 g/kg

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

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

V. Classification of the Compound:

_____Insecticide _____Herbicide _____Fungicide

_____Microbial Agent _____Economic Poison

Other: Halogenated flame retardant

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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.