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December 7, 2006

TSCA Confidential Business Information Center (7407M)
EPA EAST - Room 6428 Attn: Section 8(e)
United States Environmental Protection Agency
1201 Constitution Avenue, NW
Washington DC 20460-0001

CONTAIN NO CBI

Attention: TSCA 8(e) Coordinator

RE: Diisobutylene (CASRN 25167-70-8) – Acute Toxicity Test with the Fathead Minnow

Dear Sir or Madam:

Lyondell Chemical Company (Lyondell) hereby submits this letter pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide because it includes findings that EPA may consider reportable. The acute toxicity of Lyondell's diisobutylene product (CASRN 25167-70-8) was determined using OECD Guideline 203, "96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*). The test found the lethal loading concentration for 96 hour exposure (96-Hour LL_{50}) to diisobutylene to be 27 mg/L.

This study was conducted for Lyondell Chemical Company by Wildlife International, Ltd. in Easton, Maryland in accordance with our product stewardship program for new products.

A copy of the study final report is enclosed for your review.

Should you have any questions or require additional details, please do not hesitate to call me at 713-309-7884. I may also be reached by facsimile at 713-951-1574 or by e-mail at timothy.yagley@lyondell.com.

Sincerely,

Timothy Yagley
Business Consultant – Chemical Control
Corporate TSCA Coordinator
Lyondell Chemical Company

Enc.

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DIISOBUTYLENE:
A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 620A-103A

OECD GUIDELINE 203
U.S. EPA OPPTS NUMBER 850.1075

AUTHORS:

Susan J. Palmer
Cary A. Sutherland
Timothy Z. Kendall
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: January 4, 2006

STUDY COMPLETION DATE: November 15, 2006

SUBMITTED TO:

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Houston, TX 77010

Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10(d)(1)(A), (B) or (C).

Company: _____ (Typed Name)

Company Agent: _____ (Typed Name)

Title: _____

Signature: _____ Date: _____

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Lyondell

TITLE: Diisobutylene: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 620A-103A

STUDY COMPLETION: November 15, 2006

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792, 17 August 1989), and OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17), with the following exceptions:

Periodic analyses of well water for potential contaminants were performed using a certified laboratory and standard U.S. EPA analytical methods.

The characterization of the test substance, and the stability of the test substance under the conditions of storage at the test site, were not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Susan J Palmer — 15 November 2006

Susan J. Palmer
Senior Biologist

Date

SPONSOR APPROVAL:

Sponsor Representative — Date

Applicant/Submitter — Date

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792, 17 August 1989), and OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17). 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:
Protocol	December 12 & 16, 2005	December 16, 2005	January 4, 2006
Test Substance Preparation	September 27, 2006	September 27, 2006	October 3, 2006
Observations and Water Chemistry	September 29, 2006	September 29, 2006	October 9, 2006
Calibration Standard Preparation	September 29, 2006	September 29, 2006	October 9, 2006
Biological Data and Draft Report	October 7 & 8, 2006	October 9, 2006	October 20, 2006
Analytical Data and Draft Report	October 9, 2006	October 9, 2006	October 10, 2006
Final Report	November 15, 2006	November 15, 2006	November 15, 2006

All inspections were study-based unless otherwise noted.

Marshall T. Hynson Date 11/15/2006
Quality Assurance Program Supervisor

REPORT APPROVAL

SPONSOR: Lyondell

TITLE: Diisobutylene: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 620A-103A

STUDY DIRECTOR:

Susan J. Palmer — 15 November 2006
Susan J. Palmer
Senior Biologist
Date

PRINCIPAL INVESTIGATOR:

Timothy Z. Kendall — 11-15-06
Timothy Z. Kendall, M.S.
Supervisor
Date

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Henry O. Krueger, Ph.D.
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11/15/06
Willard B. Nixon, Ph.D.
Director of Chemistry
Date

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SUMMARY

SPONSOR: Lyondell

TITLE: Diisobutylene: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 620A-103A

TEST DATES:

Study Initiation:	January 4, 2006
Experimental Start (OECD):	September 17, 2006
Experimental Start (EPA):	September 25, 2006
Biological Termination:	September 29, 2006
Experimental Termination:	September 29, 2006

LENGTH OF EXPOSURE: 96 Hours

TEST ORGANISMS: Fathead minnow (*Pimephales promelas*)

SOURCE OF TEST ORGANISMS: Chesapeake Cultures, Inc.
Hayes, Virginia 23072

AGE OF TEST ORGANISMS: Juveniles

MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:

Mean Wet Weight: 0.13 g	Range: 0.10 – 0.17 g
Mean Total Length: 2.6 cm	Range: 2.5 – 2.8 cm

TEST CONCENTRATIONS: Since diisobutylene is known to have low water solubility, the test organisms were exposed to Water Accomodated Fractions (WAF) prepared by mixing DIB in water (c. 24 h + 4 h settling period) at the following nominal concentrations (WAF loading rates):

Nominal	Mean Measured
Negative Control	< LOQ
0.41 mg/L	< LOQ
1.4 mg/L	0.27 mg/L
4.5 mg/L	0.73 mg/L
15 mg/L	1.7 mg/L
50 mg/L	6.4 mg/L

RESULTS: Results based on nominal WAF loading rates, with results based on mean measured concentrations of the water soluble fraction presented parenthetically:

96-Hour LL50:	27 mg/L (3.3 mg/L)
95% Confidence Interval:	15 – 50 mg/L (1.7 – 6.4 mg/L)
No-Mortality Level:	15 mg/L (1.7 mg/L)
No-Observed-Effect Level:	15 mg/L (1.7 mg/L)

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Lyondell at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. An initial limit test was initiated on September 18, 2006. However, there was 100% mortality in the limit concentration of 100 mg/L within 24 hours. Therefore, the study design was changed to a dose-response test. The in-life phase of the definitive toxicity test was conducted from September 25 to 29, 2006. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 620A-103A in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute effects of diisobutylene on the fathead minnow (*Pimephales promelas*) during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Since diisobutylene is known to have low water solubility, test organisms were exposed to five water accommodated fractions (WAF) of the test substance, as well as a negative control (dilution water) for 96 hours. Nominal WAF loading rates were selected in consultation with the Sponsor based upon the results of the initial limit test, and were 0.41, 1.4, 4.5, 15 and 50 mg diisobutylene/L. Two replicate test chambers were maintained in each treatment and control group, with 10 minnows in each test chamber for a total of 20 minnows per concentration. The test chambers were sealed with minimal headspace to reduce the volatilization of the test substance. Test solutions were renewed daily by transferring the test organisms to freshly prepared solutions. Mean measured test concentrations were determined from samples of test water collected from newly prepared test solutions at the beginning of the test, and from 24-hour old test solutions at 24 and 96 hours.

Fathead minnows were impartially assigned to exposure chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 2.5, 24, 48, 72 and 96 hours after test initiation. The cumulative percent mortality observed in the treatment groups was used to determine LL50 values (i.e., the theoretical WAF loading rate that produces 50% mortality) at 24, 48, 72 and 96 hours. The no-mortality level and the no-observed-effect level (NOEL) were determined by visual interpretation of the mortality and observation data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Diisobutylene: A 96-Hour Static-Renewal Acute Toxicity Test with the Fathead minnow (*Pimephales promelas*)". The study was performed based on guidelines provided in OECD Guideline for Testing of Chemicals, 203: *Fish, Acute Toxicity Test* (1) and the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine* (2).

Test Substance

The test substance used to prepare the test solutions, analytical calibration standards and the analytical matrix fortification samples for the study was received from Lyondell on September 20, 2005. It was assigned Wildlife International, Ltd. identification number 7355 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as: Diisobutylene; Lot number GCRD10401R. The test substance had an expiration date of September 30, 2006.

Test Organism

The fathead minnow, *Pimephales promelas*, was selected as the test species for this study. Fathead minnows are representative of an important group of aquatic vertebrates, and were selected for use in the test based upon past history of use in the laboratory. Fathead minnow juveniles used in the test were obtained from Chesapeake Cultures, Inc., Hayes, Virginia. Identification of the species was verified by the supplier.

All fish used in the test were from the same source and year class, and the length of the longest fish measured was no more than twice the length of the shortest. The average total length of the 10 negative control fish measured at the end of the test was 2.6 cm, with a range of 2.5 to 2.8 cm. The average wet weight (blotted dry) of the 10 negative control fish measured at the end of the test was 0.13 grams, with a range of 0.10 to 0.17 grams. Loading was defined as the total wet weight of fish per liter of test water and was 0.33 g fish/L.

The fish were held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. During the two-week period preceding the test, water temperatures in the cultures ranged from 21.7 to 22.3°C, measured with a hand-held

liquid-in-glass thermometer. The pH of the water ranged from 8.2 to 8.6, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 7.8 to 8.4 mg/L (≥90% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter. During the holding period, the fish were fed daily a commercially-prepared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania, supplemented with brine shrimp nauplii (*Artemia* sp.) supplied by Brine Shrimp Direct, Ogden, Utah. The fish were not fed for at least two days prior to the test or during the test.

During the 14-day period prior to the test the fish showed no signs of disease or stress. At test initiation, the fathead minnows were collected from the culture tank and impartially distributed one and two at a time to the test chambers until each contained 10 fish.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH of the well water during the four-week period immediately preceding the test are presented in Appendix 1.

The well water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water was filtered to 0.45 µm to remove fine particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 2.

Test Apparatus

Test chambers were 1 gallon (approximately 4 L) glass jars with Teflon®-lined caps, and were completely filled with test solution to minimize headspace. The depth of the test water in a representative test chamber was 23.1 cm. Each test chamber was labeled with the project number, test concentration and replicate. The chambers were indiscriminately positioned in an environmental chamber designed to maintain the desired test temperature throughout the test period.

Preparation of Test Concentrations

The test solutions were prepared as water accommodated fractions (WAF) at nominal loading rates of 0.41, 1.4, 4.5, 15 and 50 mg/L. A single WAF was prepared for each concentration by weighing the test substance and adding it to 12 L of dilution water (well water) in a 13.2 L Pyrex® aspirator bottle. Each solution was stirred with a Teflon®-coated stir bar on a magnetic stir plate for approximately 24 hours. Care was taken to maintain a vortex depth approximately 30% of the test solution height. Following the mixing period, each WAF solution was allowed to settle for four hours before the solution was decanted into the test chambers through a spigot and tubing placed approximately 2-3 cm from the bottom of the aspirator bottle. After settling, the solutions in the WAF bottles appeared clear and colorless. The solutions in the test chambers appeared clear and colorless at test initiation and termination.

Analytical Sampling

Samples were collected from the batches of test solution prepared at test initiation for each treatment and control group to measure concentrations of the test substance. Samples of the 24-hour old solutions were pooled from each replicate of each treatment and control group at 24 hours and at test termination. An additional sample was collected from the 50 mg/L level on Day 2 when 100% mortality occurred in both replicates. Sampling then was discontinued in the 50 mg/L level. All samples were collected at mid-depth, placed in Teflon® centrifuge tubes, and processed immediately for analysis.

Analytical Method

The method used for the analysis of the diisobutylene samples was based on methodology developed by Wildlife International, Ltd. The analytical method consisted of fortifying recovery samples with the appropriate diisobutylene stock solution, diluting all samples with hexane, vortexing the samples, making secondary dilutions (as necessary) and transferring to autosampler vials for analysis by gas chromatography with mass selective detection.

Concentrations of diisobutylene in the samples were determined using a Hewlett-Packard 5890 Gas Chromatograph (GC) equipped with a Mass Selective Detector (MS). Chromatographic separations were achieved using a Phenomenex ZB-624 column (30 m x 0.25 mm, 1.4-µm film

thickness). A flowchart for the analysis of diisobutylene is provided in Appendix 3.1 and the instrumental parameters for the analysis are provided in Appendix 3.2.

Calibration standards of diisobutylene, ranging in concentration from 0.200 to 2.00 mg/L, were prepared in hexane using a stock solution of diisobutylene test substance in hexane (Appendix 3.3). Fresh calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the sum of the peak area responses versus the respective concentrations of the calibration standards. The concentration of diisobutylene in the samples was determined by substituting the sum of the peak area responses of the samples into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 3.4.

The method limit of quantitation (LOQ) for the analysis of diisobutylene in freshwater was set at 0.200 mg/L, calculated as the product of the concentration of the lowest calibration standard (0.200 mg/L) and the dilution factor of the matrix blank samples (1.00). Three matrix blank samples were analyzed during the test to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 3.5).

Samples of freshwater were fortified at 0.400, 1.00 and 2.00 mg/L using stock solutions of diisobutylene in tetrahydrofuran (Appendix 3.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples for diisobutylene ranged from 82.2 to 97.4% of nominal concentrations (Appendix 3.5).

A representative calibration curve is presented in Appendix 3.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. Representative chromatograms of a freshwater matrix blank sample and a matrix fortification sample are presented in Appendices 3.9 and 3.10, respectively. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the cultures and test chambers. A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light

intensity was provided at the beginning and end of the 16-hour light period to avoid sudden changes in lighting. Light intensity at test initiation, measured using a SPER Scientific Model 840006C light meter, was 453 lux at the surface of the water of one representative test chamber.

The target test temperature during the study was 22 ± 2°C. Temperature was measured continuously during the test using a Fulscope ER/C Recorder in a container of water placed adjacent to the test chambers. The continuous recorder was verified prior to test initiation with a liquid-in-glass thermometer. Temperature, dissolved oxygen and pH were measured in the WAF solutions prepared for each treatment and control group at test initiation and at approximately 24, 48, 72 and 96 hours, and in the 24-hour old test solution in each test chamber at 24, 48, 72 and 96 hours. Temperature measurements were made using a hand-held liquid in glass thermometer. Dissolved oxygen was measured using a Thermo Orion Model 850Aplus dissolved oxygen meter and measurements of pH were made using a Thermo Orion Model 525Aplus pH meter. When 100% mortality occurred in a test chamber, measurements of temperature, dissolved oxygen and pH were taken and then discontinued.

Specific conductance, hardness and alkalinity were measured in the dilution water at the beginning of the test. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (3).

Observations

Observations were made periodically to determine the number of mortalities in each treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior were also evaluated. Observations were made approximately 2.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The mortality data were analyzed using the computer program of C. E. Stephan (4). The program was designed to calculate the LL50 value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (5, 6, 7). In this study, the binomial probability method was used to calculate the 48, 72 and 96-hour LL50

values. There were no mortalities in any treatment group at 24 hours. Therefore, the 24-hour LL50 value, as well as the no-mortality level and NOEL, were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal WAF loading rates selected for use in this study were 0.41, 1.4, 4.5, 15 and 50 mg diisobutylene/L. Results of analyses to measure concentrations of diisobutylene in the test solution samples collected during the test are presented in Table 1. Samples collected during the test from the 0.41 mg/L nominal loading rate had measured concentrations that were <LOQ. Samples collected from the 1.4, 4.5, 15 and 50 mg/L nominal loading rates has measured concentrations that ranged from 10 to 27% of nominal concentrations, with the exception of the 96-hour sample collected from the 1.4 mg/L nominal loading rate. The measured concentration in the 96-hour sample was <LOQ. When the measured concentrations of samples collected at nominal loading rates of 1.4, 4.5, 15 and 50 mg/L were averaged, the mean measured concentrations for the study were 0.27, 0.73, 1.7 and 6.4 mg/L, representing 19, 16, 11 and 13% of nominal WAF loading rates, respectively. The results of the study are based on the nominal WAF loading rates, with results based on the mean measured concentrations of the water-soluble fraction presented parenthetically.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH in the test solutions are presented in Table 2. Water temperatures were within the 22 ± 2°C range established for the test. Dissolved oxygen concentrations in the newly prepared test solutions were ≥7.9 mg/L (≥91% of saturation). Dissolved oxygen concentrations measured in the 24-hour old solutions collected from the closed-bottle test chambers ranged from 4.1 to 6.6 mg/L (47 to 76% of saturation). Measurements of pH ranged from 7.8 to 8.2. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations of mortality and signs of toxicity observed during the test are presented in Table 4. All fathead minnows in the negative control group appeared normal throughout the test. All fathead minnows exposed to diisobutylene at nominal WAF loading rates ≤15 mg/L (1.7 mg/L based on mean measured concentrations) also appeared normal throughout the test, with no mortalities or

overt signs of toxicity observed. All fish exposed at a nominal WAF loading rate of 50 mg/L (6.4 mg/L) were dead within 48 hours of exposure. The no-mortality level and the NOEL were both 15 mg/L (1.7 mg/L). LL50 values at 24, 48, 72 and 96 hours were determined from the mortality data and are shown in Table 5. A graph of the concentration-response curve is included in Figure 1.

Conditions for Validity of the Test

The following criteria used to judge the validity of the test were met, with the stated exception:

1) mortality of the fish in the negative control group did not exceed 10% by the end of the test;
2) the dissolved oxygen concentration was at least 60% of the air-saturation value in the new test solutions prepared daily during the test. However, dissolved oxygen concentrations in the 24-hour old solutions collected from the closed-bottle test chambers ranged from 4.1 to 6.6 mg/L (47 to 76% of saturation).

CONCLUSIONS

Fathead minnows, *Pimephales promelas*, were exposed for 96 hours under static-renewal conditions to five nominal WAF loading rates of diisobutylene, ranging from 0.41 to 50 mg/L (<LOQ to 6.4 mg/L mean measured concentrations). The 96-hour LL50 value was 27 mg/L (3.3 mg/L based on mean measured concentrations), with a 95% confidence interval of 15 to 50 mg/L (1.7 to 6.4 mg/L). Fathead minnows exposed to diisobutylene at nominal WAF loading rates ≤15 mg/L (1.7 mg/L) appeared normal with no mortality or overt signs of toxicity. The no-mortality level and NOEL were both 15 mg/L (1.7 mg/L).

REFERENCES

1 **Organization for Economic Cooperation and Development.** 1992. *Fish, Acute Toxicity Test.* OECD Guideline for Testing of Chemicals. Guideline 203. Paris.

2 **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine.*

3 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

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. Pp. 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

Measured Concentrations of Diisobutylene in Freshwater Samples

Nominal WAF Loading Rate (mg/L)	Sample Number (620A-103A-)	Sampling Time (Hours)	Measured Concentration (mg/L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg/L)	Mean Measured Percent of Nominal
Negative Control (0.0)	1	0	< LOQ	--	< LOQ	--
	7	24	< LOQ	--		
	14	96	< LOQ	--		
0.41	2	0	< LOQ	--	< LOQ	--
	8	24	< LOQ	--		
	15	96	< LOQ	--		
1.4	3	0	0.380	27.2	0.27	19
	9	24	0.334	23.9		
	16	96	< LOQ[3]	--		
4.5	4	0	0.900	20.0	0.73	16
	10	24	0.829	18.4		
	17	96	0.456	10.1		
15	5	0	1.69	11.3	1.7	11
	11	24	1.61	10.7		
	18	96	1.72	11.5		
50	6	0	5.85	11.7	6.4	13
	12	24	6.02	12.0		
	13	48	7.32	14.6		

[1] The limit of quantitation (LOQ) was 0.200 mg/L, calculated as the product of the concentration of the lowest calibration standard (0.200 mg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

[3] One-half the LOQ was selected as a conservative estimate for use in the calculation of the mean measured concentration.

Table 2

Temperature, Dissolved Oxygen and pH in the Test Solutions

Nominal WAF Loading Rate (mg/L)	Replicate	0 Hour			24 Hours						48 Hours		
		New Solution [1]			Old Solution [2]			New Solution [1]			Old Solution [2]		
		Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH
Negative Control	A	21.9	8.1	8.2	22.2	4.7	8.0	22.0	8.3	8.2	22.2	5.8	7.9
	B				22.1	4.6	7.9				22.2	5.5	8.0
0.41	A	22.2	8.0	8.2	22.2	5.3	8.0	22.4	8.1	8.1	22.2	5.7	7.9
	B				22.2	4.8	8.0				22.2	5.7	8.0
1.4	A	22.2	8.0	8.2	22.2	4.8	8.0	22.4	8.1	8.1	22.2	5.0	7.9
	B				22.1	4.7	7.9				22.2	5.6	8.0
4.5	A	22.3	8.0	8.2	22.2	4.3	7.9	22.4	8.0	8.1	22.2	4.7	7.9
	B				22.2	4.5	7.9				22.2	4.8	8.0
15	A	22.6	8.0	8.2	22.2	4.6	7.9	22.4	8.0	8.1	22.2	4.9	7.9
	B				22.2	4.6	8.0				22.2	4.6	7.9
50	A	22.5	8.0	8.2	22.2	4.6	7.9	22.1	8.3	8.1	22.2	6.6	7.8
	B				22.1	4.5	7.9				22.2	6.1	7.9

[1] New Solution = measured in the newly prepared WAF solutions.
[2] Old Solution = measured in the 24-hour old solutions collected from the test chambers.
[3] Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 22.0 to 22.5°C, measured to the nearest 0.5°C.
[4] A dissolved oxygen concentration of 5.2 mg/L represents 60% saturation at 22°C in freshwater.

Table 2 (Continued)

Temperature, Dissolved Oxygen and pH in the Test Solutions

Nominal WAF Loading Rate (mg/L)	Replicate	48 Hours			72 Hours						96 Hours		
		New Solution [1]			Old Solution [2]			New Solution [1]			Old Solution [2]		
		Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH	Temp.[3] (°C)	DO[4] (mg/L)	pH
Negative Control	A	21.8	8.2	8.1	22.0	5.7	7.9	22.0	8.0	8.2	22.2	6.0	7.9
	B				22.0	5.7	7.9				22.2	6.0	7.9
0.41	A	22.1	8.0	8.2	22.2	6.5	7.9	22.2	8.0	8.2	22.2	5.7	7.9
	B				22.2	6.0	7.9				22.2	5.6	8.0
1.4	A	22.3	8.0	8.2	22.2	5.0	7.9	22.3	7.9	8.2	22.2	4.7	7.9
	B				22.2	5.0	7.9				22.2	4.1	7.9
4.5	A	22.5	8.1	8.1	22.2	5.1	7.9	22.1	8.1	8.2	22.2	4.5	7.9
	B				22.2	5.2	7.9				22.2	4.3	7.9
15	A	22.6	7.9	8.1	22.2	5.0	7.9	22.1	8.0	8.2	22.2	5.7	8.0
	B				22.2	4.6	7.9				22.2	5.6	8.0
50	A	--[5]	--[5]	--[5]	--[5]	--[5]	--[5]	--	--	--	--	--	--
	B				--[5]	--[5]	--[5]	--	--	--	--	--	--

[1] New Solution = measured in the newly prepared WAF solutions.
[2] Old Solution = measured in the 24-hour old solutions collected from the test chambers.
[3] Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 22.0 to 22.5°C, measured to the nearest 0.5°C.
[4] A dissolved oxygen concentration of 5.2 mg/L represents 60% saturation at 22°C in freshwater.
[5] Measurements were discontinued after 48 hours due to 100% mortality.

Table 3

Hardness, Alkalinity and Specific Conductance
Measured in Dilution Water at Test Initiation

Parameter	Day 0
Hardness (mg/L as $CaCO_3$)	132
Alkalinity (mg/L as $CaCO_3$)	178
Specific Conductance (µmhos/cm)	330

Table 4

Cumulative Mortality and Observations

Nominal WAF Loading Rate (mg/L)	Replicate	No. Exposed	2.5 Hours		24 Hours [3]		48 Hours [3]		72 Hours [3]		96 Hours		Cumulative Percent Mortality
			No. Dead[1]	Obs.[2]	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
0.41	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
1.4	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
4.5	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
15	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
50	A	10	0	10 AN	0	2 A; 8 AN	10	--	10	--	10	--	100
	B	10	0	10 AN	0	10 AN	10	--	10	--	10	--	

[1] Cumulative number of dead fish.
[2] Observations: AN = appear normal; A = surfacing.
[3] Test solutions were renewed at approximately 24-hour intervals.

Table 5

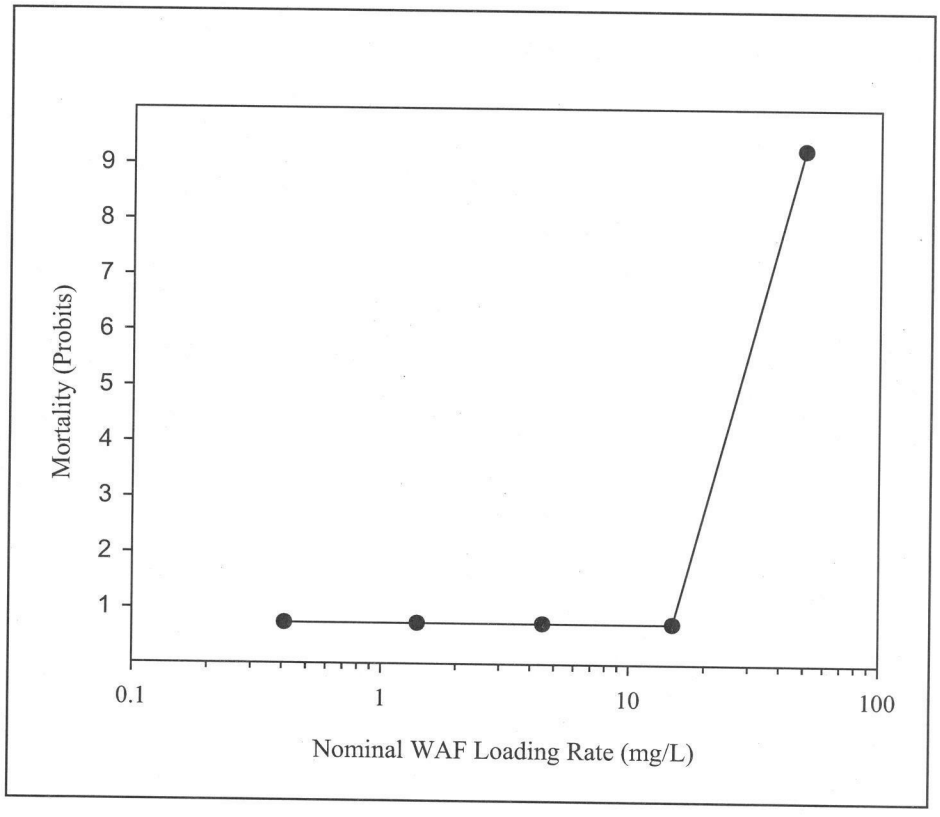
LL50 Values [1]

Time	LL50 (mg/L)	95% Confidence Interval (mg/L)	Statistical Method
24 Hours	>50 (>6.4)	--[2]	NA [3]
48 Hours	27 (3.3)	15 – 50 (1.7 – 6.4)	Binomial Probability
72 Hours	27 (3.3)	15 – 50 (1.7 – 6.4)	Binomial Probability
96 Hours	27 (3.3)	15 – 50 (1.7 – 6.4)	Binomial Probability

[1] Results were calculated using the nominal WAF loading rates, with results calculated using the mean measured concentrations presented parenthetically.
[2] 95% confidence limits could not be calculated with the mortality data obtained.
[3] NA = not applicable; the absence of mortality at 24 hours precluded statistical calculation of an LL50 value.

Figure 1

Concentration-Response Curve (96-Hour Mortality Data)



Appendix 1

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Specific Conductance (µmhos/cm)	311 (N = 4)	300 – 325
Hardness (mg/L as $CaCO_3$)	137 (N = 4)	136 – 140
Alkalinity (mg/L as $CaCO_3$)	182 (N = 4)	181 – 182
pH	8.0 (N = 4)	8.0 – 8.1

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water [1]

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.019	Heptachlor	< 0.0096
Alpha BHC	< 0.0096	Heptachlor Epoxide	< 0.0096
Alpha Chlordane	< 0.0096	Kepone	< 0.19
Beta BHC	< 0.038	Malathion	< 1.9
Bolstar	< 1.9	Merphos	< 1.9
Chlordane	< 0.48	Methoxychlor	< 0.096
Coumaphos	< 2.9	Methyl Parathion	< 1.9
Delta BHC	< 0.0096	Mevinphos	< 1.9
Demeton-O	< 1.9	Mirex	< 0.11
Demeton-S	< 1.9	Naled	< 2.9
Diazinon	< 1.9	o,p-DDD	< 0.019
Dichlorvos	< 1.9	o,p-DDE	< 0.019
Dieldrin	< 0.029	o,p-DDT	< 0.019
Disulfoton	< 1.9	p,p-DDD	< 0.019
Dursban (Chlorpyrifos)	< 1.9	p,p-DDE	< 0.019
Endosulfan I	< 0.0096	p,p-DDT	< 0.019
Endosulfan II	< 0.019	PCB-1016	< 0.48
Endosulfan Sulfate	< 0.019	PCB-1221	< 0.48
Endrin	< 0.019	PCB-1232	< 0.48
Endrin Aldehyde	< 0.096	PCB-1242	< 0.48
Endrin Ketone	< 0.019	PCB-1248	< 0.48
EPN	< 3.8	PCB-1254	< 0.48
Ethion	< 1.9	PCB-1260	< 0.48
Ethoprop	< 1.9	Phorate	< 1.9
Ethyl Parathion	< 1.9	Ronnel	< 1.9
Famphur	< 1.9	Stirophos	< 1.9
Fensulfothion	< 3.8	Telodrin	< 0.0096
Fenthion	< 1.9	Tokuthion	< 1.9
Gamma BHC – Lindane	< 0.0096	Toxaphene	< 0.96
Gamma Chlordane	< 0.096	Trichloronate	< 1.9
Guthion (Azinphos-methyl)	< 3.8	Trithion	< 1.9
HCB	< 0.096		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 15, 2005.

Appendix 2 (Continued)

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Metals			
Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum	< 0.200	Magnesium	13.3
Antimony	< 0.0200	Manganese	< 0.0050
Arsenic	< 0.0200	Mercury	< 0.00020
Barium	< 0.0050	Nickel	< 0.0100
Beryllium	< 0.0050	Nitrate Nitrogen	< 0.50
Bromide	< 2.5	Nitrite Nitrogen	< 0.50
Cadmium	< 0.0050	Potassium	7.65
Calcium	33.1	Selenium	< 0.0200
Chloride	2.7	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.1
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	0.56	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0200		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 15, 2005.

Appendix 3

The Analysis of Diisobutylene in Freshwater

Appendix 3.1

Analytical Method Flowchart for the Analysis of Diisobutylene in Freshwater

FLOWCHART FOR THE ANALYSIS OF DIISOBUTYLENE IN FRESHWATER

Rinse all Teflon® centrifuge tubes with hexane.

↓

Add 10.0 mL of freshwater to each centrifuge tube.

↓

Fortify recovery samples with the appropriate Diisobutylene stock solution. Unfortified freshwater will serve as the matrix blank.

↓

Add 10.0 mL of hexane to each sample. Vortex each sample for 15 seconds. If appropriate, make secondary dilutions in hexane.

↓

Transfer an aliquot of the extract to an autosampler vial and submit for analysis by GC/MS.

Appendix 3.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 5890 Gas Chromatograph (GC)
DETECTOR:	Hewlett-Packard Model 59714A Mass Selective Detector operated in SIM (Single Ion Monitoring) mode
ANALYTICAL COLUMN:	Phenomenx ZB-624 column (30 m x 0.25 mm, 1.4-µm film thickness)
INJECTOR TEMPERATURE:	150°C
OVEN:	Initial temperature: 45°C Initial hold time: 10 minutes Ramp: 25°C/minute Final temperature: 110°C Final hold time: 2.40 minutes
DETECTOR TEMPERATURE:	280°C
INJECTION VOLUME:	2.0 µL; splitless
CARRIER GAS:	Helium
HEAD PRESSURE:	21 kPa
ION MONITORED:	57.00 m/z
APPROXIMATE DIISOBUTYLENE RETENTION TIMES:	8.6 and 11.7 minutes

Appendix 3.3

Analytical Stocks and Standards Preparation

A stock solution of diisobutylene was prepared by weighing 0.01000 g of the test substance on an analytical balance. The test substance was transferred to a 100-mL Class A volumetric flask and brought to volume using tetrahydrofuran. The primary stock solution (0.100 mg/mL) was used to fortify the quality control samples.

A second stock solution of diisobutylene was prepared by weighing 0.1004 g of the test substance on an analytical balance. The test substance was transferred to a 100-mL Class A volumetric flask and brought to volume using hexane. The primary stock solution (1.00 mg/mL) was diluted with hexane to prepare a 0.100 mg/mL stock solution which was used to prepare the calibration standards in hexane. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg/mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg/L)
0.100	20.0	10.0	0.200
0.100	50.0	10.0	0.500
0.100	100	10.0	1.00
0.100	150	10.0	1.50
0.100	200	10.0	2.00

Appendix 3.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 620A-103A-3, with a nominal concentration of 1.4 mg/L, were calculated using the following equations:

$$\text{Concentration of Diisobutylene in sample (mg/L)} = \frac{\text{Sum of peak areas - (y-intercept)}}{\text{slope}} \text{ x dilution factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{Measured concentration of sample (mg/L)}}{\text{Nominal concentration of sample (mg/L)}} \text{ x 100}$$

Sum of Peak Areas = 1086700
Y-Intercept = -17597
Slope = 2903593
Dilution Factor = 1.00

$$\text{Concentration of Diisobutylene in sample (mg/L)} = \frac{1086700 + 17597}{2903593} \text{ x 1.00}$$

Concentration of Diisobutylene in sample (mg/L) = 0.380

$$\text{Percent of nominal concentration} = \frac{0.380 \text{ mg/L}}{0.41 \text{ mg/L}} \text{ x 100}$$

Percent of nominal concentration = 27.2%*

* Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Appendix 3.5

Quality Control Samples of Diisobutylene in Freshwater

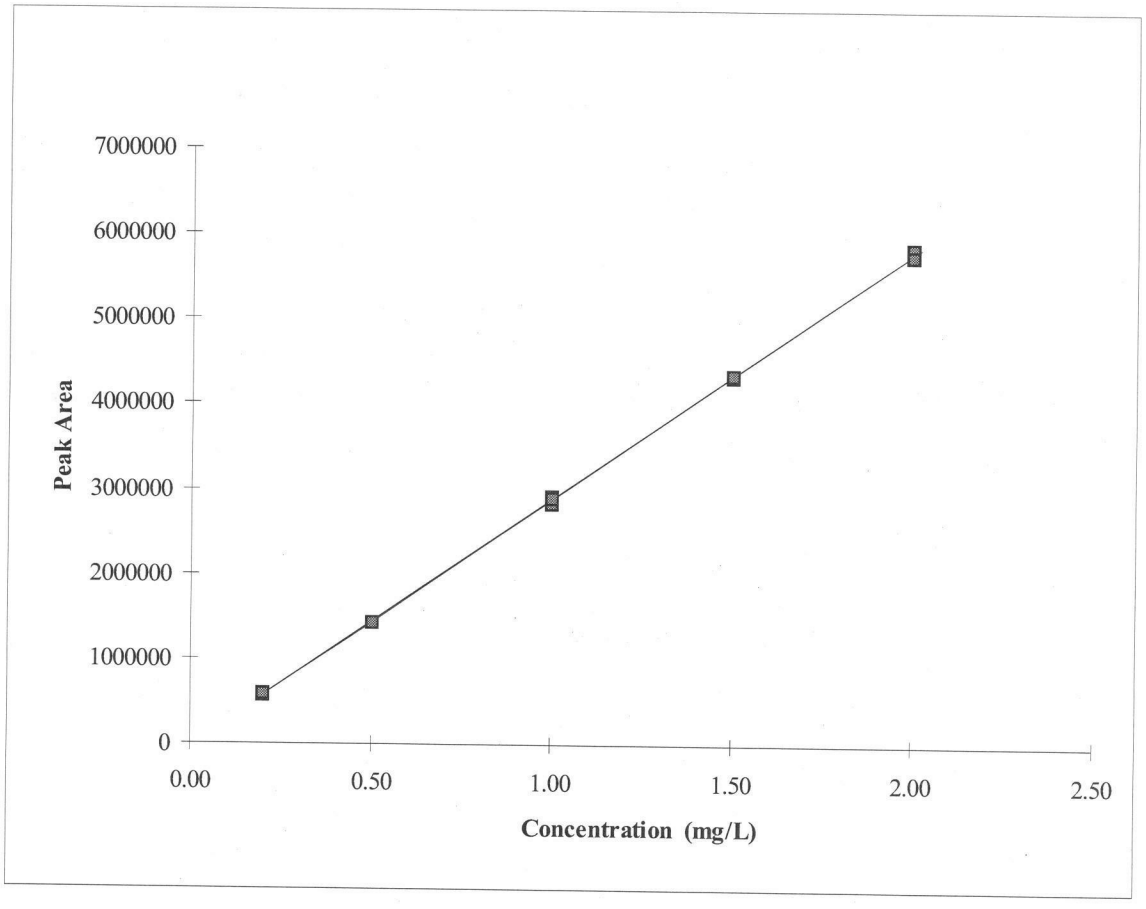
Sample Number (620A-103A-)	Sample Type	Concentration of Diisobutylene		Percent Recovery[2]
		Fortified (mg/L)	Measured[1] (mg/L)	
MAB-1	Matrix Blank	0.0	< LOQ	--
MAB-2	Matrix Blank	0.0	< LOQ	--
MAB-3	Matrix Blank	0.0	< LOQ	--
MAS-1	Matrix Fortification	0.400	0.329	82.2
MAS-2	Matrix Fortification	1.00	0.843	84.3
MAS-3	Matrix Fortification	2.00	1.71	85.6
MAS-4	Matrix Fortification	0.400	0.380	95.0
MAS-5	Matrix Fortification	1.00	0.943	94.3
MAS-6	Matrix Fortification	2.00	1.95	97.4
MAS-7	Matrix Fortification	0.400	0.357	89.2
MAS-8	Matrix Fortification	1.00	0.921	92.1
MAS-9	Matrix Fortification	2.00	1.89	94.3
			Mean =	90.5
			Standard Deviation =	5.39
			CV =	5.95%

[1] The limit of quantitation (LOQ) of 0.200 mg/L was calculated as the product of the concentration of the lowest calibration standard (0.200 mg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Appendix 3.6

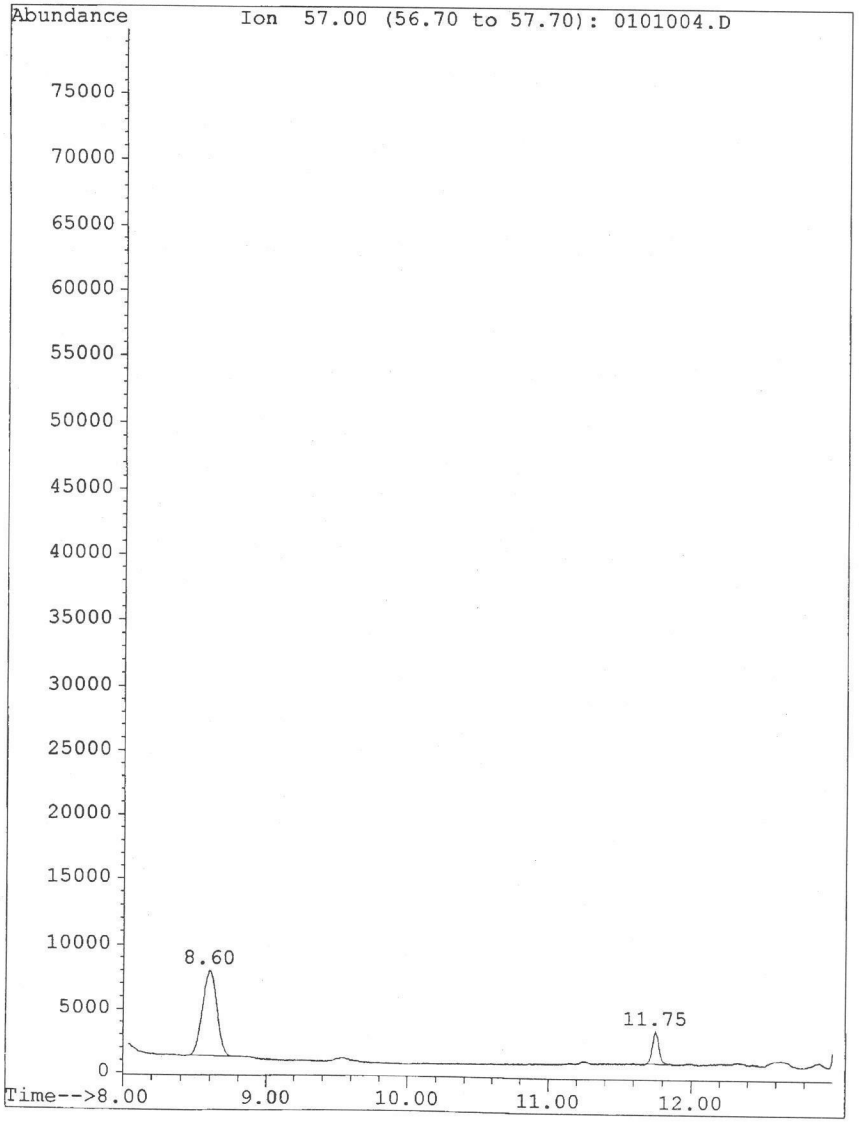
Representative Calibration Curve for Diisobutylene



Slope = 2903593; Y-Intercept = -17597; R^2 = 0.9998

Appendix 3.7

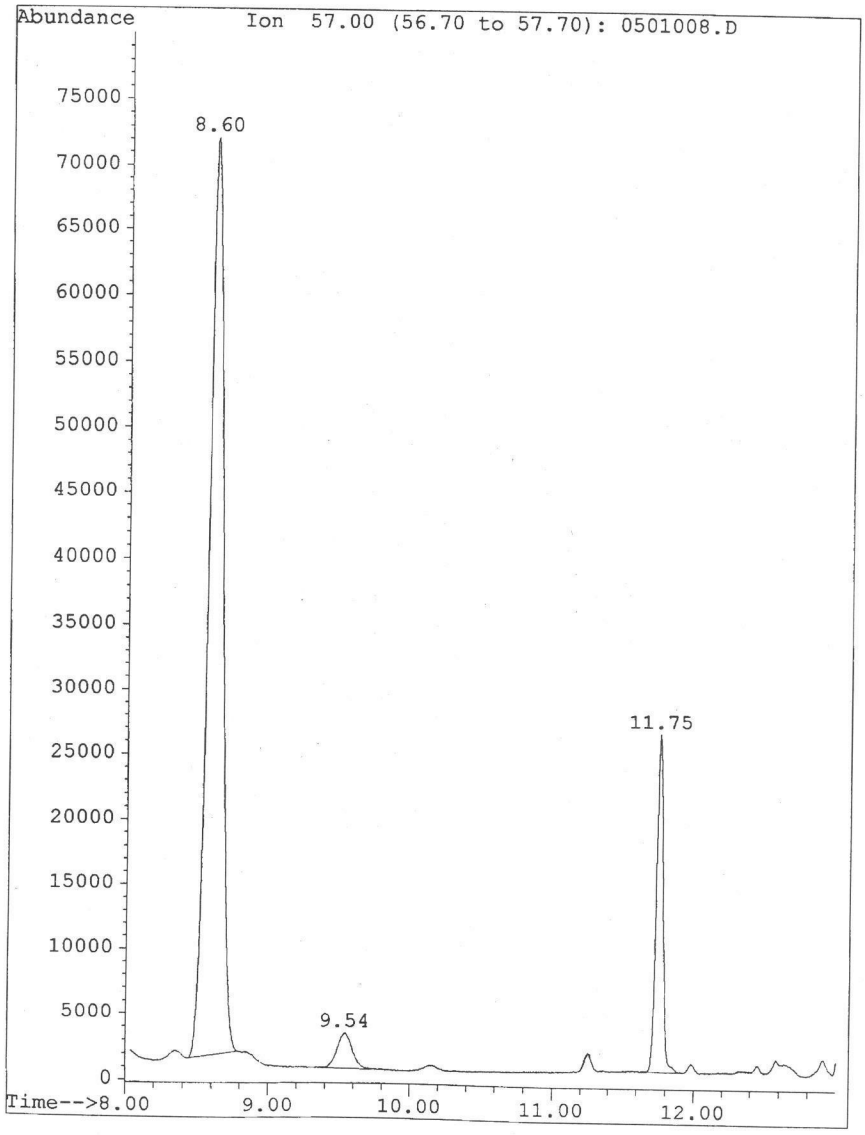
Representative Chromatogram of a Low-level Diisobutylene Calibration Standard



Nominal concentration: 0.200 mg/L

Appendix 3.8

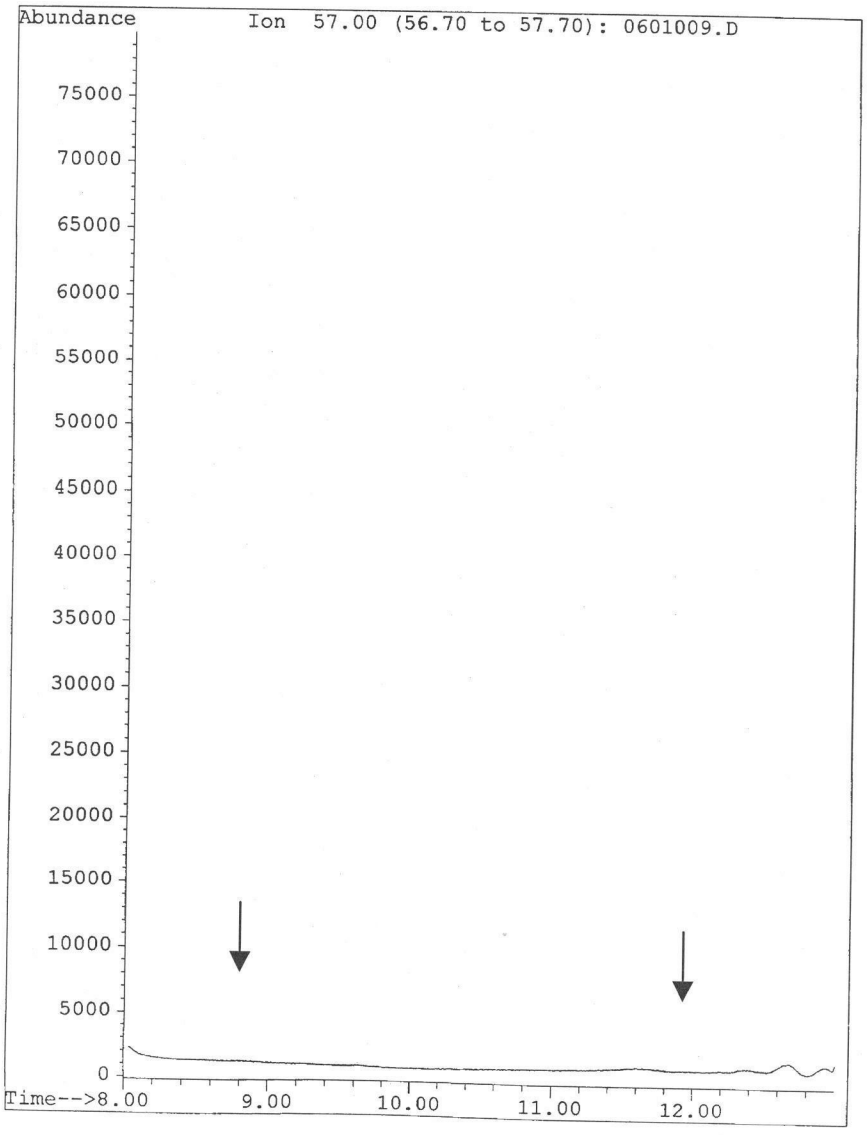
Representative Chromatogram of a High-level Diisobutylene Calibration Standard



Nominal concentration: 2.00 mg/L

Appendix 3.9

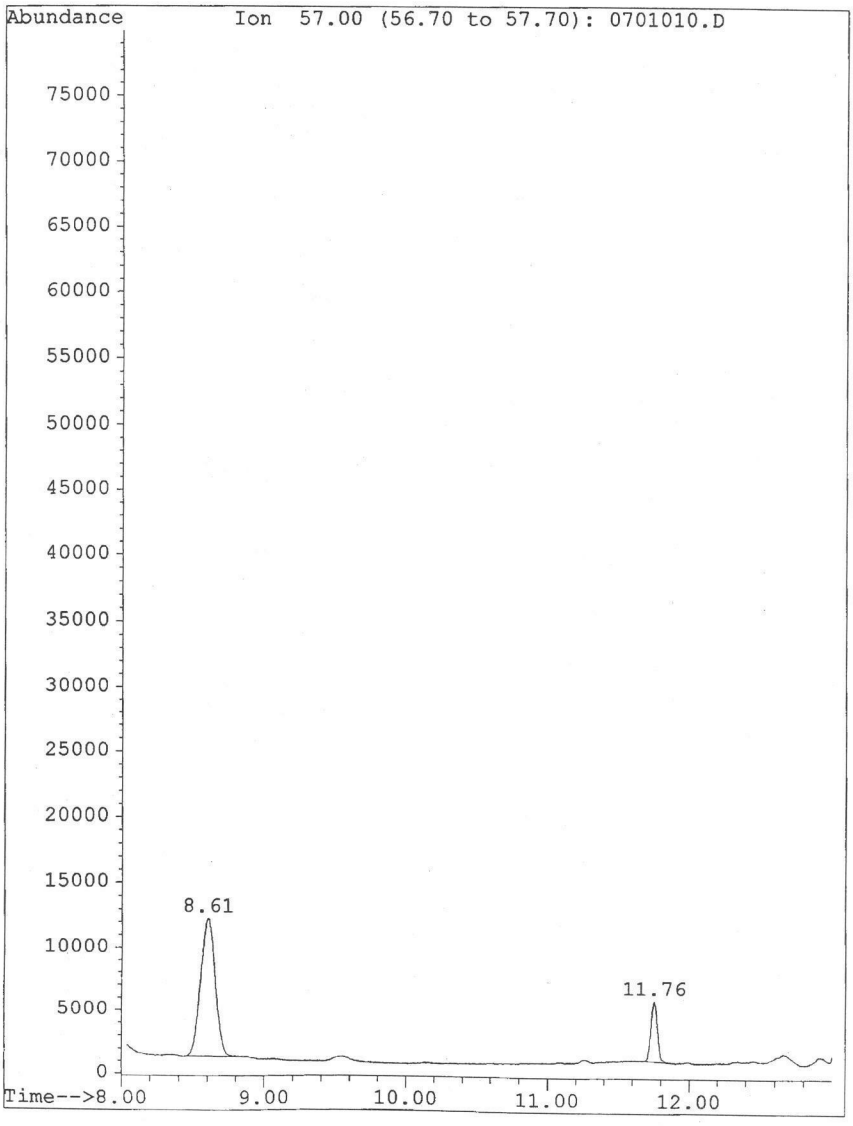
Representative Chromatogram of a Matrix Blank Sample



Sample number 620A-103A-MAB-1. The arrows indicate the approximate retention times of diisobutylene.

Appendix 3.10

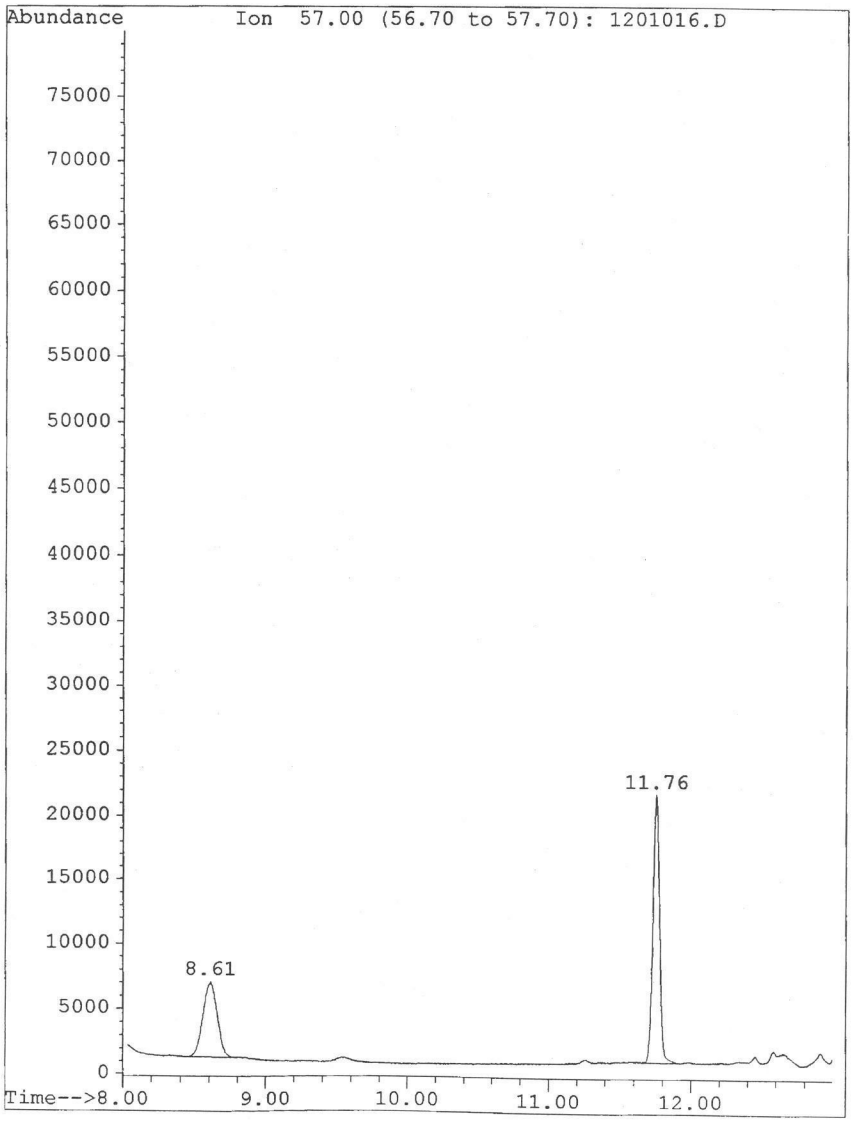
Representative Chromatogram of a Matrix Fortification Sample



Sample number 620A-103A-MAS-1; 0.400 mg/L nominal concentration

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number 620A-103A-3; Day 0; 1.4 mg/L nominal concentration

Appendix 4

Changes to Protocol

This study was conducted in accordance with the approved protocol with the following changes:

1. The protocol was amended to add the proposed test dates, the test concentrations and the test substance identification for the initial limit test.

2. The protocol was amended to change experimental design from a dose response test to a limit test.

3. The protocol was amended to add WAF mixing and settling times and to remove any reference to a WAF equilibration study.

4. The protocol was amended to change the vortex depth to 30% of the height of the solution.

5. The protocol was amended to add duplicate samples from each pooled test solution for each treatment group and control at 0, 24 and 96 hours in the initial limit test, with 0-hour samples collected from fresh WAF test solutions and 24 and 96-hour samples collected from the old test solutions.

6. The protocol was amended to change the experimental start and termination dates and the test concentrations, and to change the experimental design from a limit test to a dose-response test.

7. The protocol was amended to change the analytical sampling from duplicate samples to single samples for each test concentration and control, and to clarify sample collection at 0, 24 and 96 hours in the dose-response test.

8. The protocol was amended to reassign Study Director responsibilities.

Appendix 5

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Amy S. Blankinship, Laboratory Supervisor
4. Timothy Z. Kendall, Supervisor
5. Cary A. Sutherland, Senior Biologist
6. Susan J. Palmer, Senior Biologist